An Investigation of the Role of rel Family Members in the Early Development of *Xenopus laevis.*

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"Theories come and go. The frog remains."

Jean Rostand, 1960

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Declaration

All the results in this thesis were obtained by the author, apart from this which are specifically indicated in the text. All injections and almost all dissections of *Xenopus* embryos were performed by Professor Hugh Woodland.

All sources of information have been specifically acknowledged by means of reference. None of the work contained in this thesis has been used for any previous application for a degree.

David Sutherland

Abbreviations

APS	Ammonium persulphate
ATP, dATP	adenosine triphosphate, deoxyadensoine triphosphate
bp	base pair
BSA	bovine serum albumin
BCIP	5-bromo-4-chloro-3-indolyl-phosphate (4-toluidine salt)
Ci	Curie
CIAP	Calf intestinal alkaline phosphatase
CHAPS	3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulphonate
CTP, dCTP	cytidine triphosphate, deoxyadensosine triphosphate
DIG	digoxygenin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetatra-acetete (as tetra sodium salt)
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
FSH	follicle stimulating hormone
g	gram
GTP	guanosine triphosphate, deoxyguanosine triphophate
hCG	human chorionic gonadotrophin
HIV	human immunodeficiency virus
HSV	herpes simplex virus
l	litre
LTR	long terminal repeat
m	metre
MBT	mid-blastula transition
MOPS	3-[N-mopholino) propane-sulphonic acid
mRNA	messenger RNA
NBT	nitro blue tetrazolium chloride
OD	optical density
PAGE	poly-acrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl chloride
RPM	revolutions per minute
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N' - tetramethyl-ethylethnediamine

TK	thymidine kinase
Tris	tris(hydroxymethyl) methylamine
tRNA	transfer RNA
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
v/v	volume per volume
v/v	weight per volume

Summary

XrelA is a maternally expressed *Xenopus* member of the rel family of transcription factors which includes the *Drosophila* dorsal-ventral patterning gene *dorsal*. During the blastula stages XrelA becomes differentially localised to the nuclei of the animal hemisphere and marginal zone. Another rel family member, known as Xrel.2, which is also expressed maternally has been cloned recently, and is a likely endogenous heterodimerisation partner for XrelA. Various attempts have been made previously to investigate the developmental role of XrelA. Over-expression of XrelA in embryos has dramatic phenotypic effects, but these are probably largely due to transcriptional squelching. The effects of over-expressing a dominant inhibitory variant of XrelA lacking the transactivation domain, which acts by saturating DNA binding sites for XrelA homodimers, have also been characterised. However, the DNA binding specificity of XrelA present in XrelA/Xrel.2 heterodimers is likely to differ from this.

To avoid this problem, dominant inhibitory variants deficient in DNA binding activity, which act by sequestering wild-type protein into inactive complexes, were constructed from XrelA (XrelAΔSP) and human NFKB1 (p50ΔSP). Both clones are capable of inhibiting DNA binding and transactivation by exogenous XrelA and do not possess the squelching activity of wild-type XrelA. Three κB binding complexes have been detected in embryos, none of which contains XrelA. The activity of one of these complexes is inhibited by p50ΔSP, but not by XrelAΔSP. Expression of XrelA ΔSP in embryos has dramatic phenotypic effects. This phenotype has been analysed in detail and exhibits some similarities to that caused by the dominant negative FGF receptor. The fact that expression of p50ΔSP, or of a deletion of XrelAΔSP (XrelAΔ SP222), which lacks the transactivation domain, have no phenotypic effects suggests that the phenotype probably arises by some mechanism other than inhibition of XrelA, and that the transactivation domain is necessary to this mechanism. Future work might concentrate on the identification of factors interacting with this domain.

1. Introduction

1.1. The Rel Family of DNA binding proteins

The aim of this section is to review the current state of understanding the activities of mamalian rel family members and their control. I aim, in doing so, to provide essential background to understanding the strategy used to investigate the roles of rel family members in *Xenopus* early development.

1.1.1. Overview of the rel and IκB families.

The rel gene family encodes a group of dimeric DNA binding proteins (see figure 1 for nomenclature and relationships) containing a highly conserved domain of about 300 amino acids known as the 'rel homology domain' (RHD). The RHD contains regions necessary for DNA binding and for dimerisation with other family members. In fact conservation is such that all of the known mammalian rel family members can heterodimerise with all the others, with the exception of relB, which does not homodimerise and forms heterodimers with p50 and p52 only (Bours *et al.*, 1993). The family can be subdivided into two groups, one of which includes rel family members with C-terminal transactivation domains. The known mammalian members of this group are *RelA*, c-*rel*, and *RelB*. The other group, so far consisting of two genes called *NFKB1* and *NFKB2*, have no transactivation domains but are transcribed as large precursors with C-terminal inhibitory domains. In order to be active (i.e.:- able to translocate to the nucleus and to bind DNA) these gene products require proteolytic processing, which removes and degrades the inhibitory domain (Pallombella *et al.*, 1994). However they can dimerise in their unprocessed state and so sequester other rel family members into inactive complexes. As might be expected, the promiscuity of dimerisation of rel family members is significant as a source of variation with respect to control of activation, DNA binding specificity and affinity, and transactivation activity. The current state of knowledge of the differences in activities between rel family dimers is summarised in figure 4. Rel family dimers can be activated by a wide range of stimuli, including tumour necrosis factor α (TNF-α), interleukin-1 (IL-1), lipopolysaccaride (LPS), and phorbol myristate actetate (PMA). Models of the mechanism of activation are discussed later (see section 1.1.2.).

A further complexity arises from the fact that activation, and in some cases transactivation activity, of rel family members is controlled by a family of interacting proteins, which differ in their specificity and affinity for the various rel dimers (see figure 4), and in means of activation. These related proteins, referred to as the IκB family, all contain ankyrin-like repeat domains (ARD's) which are common motifs involved in protein-protein interaction (see Blank *et al.*, 1992 for review). The prototypical member of this family, IκBα, is specific for p65, Rel, and relB[1] containing dimers (Duckett *et al.*, 1993; Dobrzanski *et al.*, 1994) which it binds to and retains in the cytoplasm. Phosphorylation of IκBα is thought to act as a signal which marks it for degradation (possibly involving the proteasome) while still bound to the rel dimer (Didonato *et al.*, 1995). The loss of IκBα from the complex unmasks nuclear localisation signals present on the rel dimer (Beg *et al.*, 1992) and so allows the dimer to translocate to the nucleus where it can activate or repress transcription of target genes. As well as being able to retain certain rel complexes in the cytoplasm, I κBα can inhibit their DNA binding activity *in vitro* (Nolan *et al.*, 1991). Interestingly the IκBα gene itself is under the positive control of NF-κB (Le Bail *et al.*, 1993). This may be a negative feedback mechanism which allows a short burst of activation of NF-κB before active dimers are sequestered by resynthesised IκBα. This provides a neat explanation of the function of IκBα's ability to inhibt DNA binding.

Another member of the family, IκBβ, has identical specificity and inhibitory functions to IκBα (as far as has been tested), but its degradation is induced by only a subset of the factors which can induce IκBα degradation . For example, IL-1 and LPS treatment cause degradation of both whereas TNFα and PMA cause degradation of only IκBα (Thompson *et al.*, 1995). The major difference in the activation of NF-κB involving IκBβ as well as IκBα is that it is sustained for much longer (hours rather than minutes) than activaton involving IκBα alone. Unlike IκBα, the IκBβ gene is not under autoregulatory control by NF-κB. Interestingly, even in cells which contain both isoforms, activation of NF-κB from a pool bound to IκBβ is sustained for much longer than that from the IκBα bound pool (Thompson *et al.*, 1995). If IκB α is indeed involved in negative feedback inhibition of active, nuclear NF-κB, activation of IκBβ bound NF-κB must include modification (e.g.-phosphorylation) of NF-κB itself to somehow make it insensitive to inhibition by IκBα.

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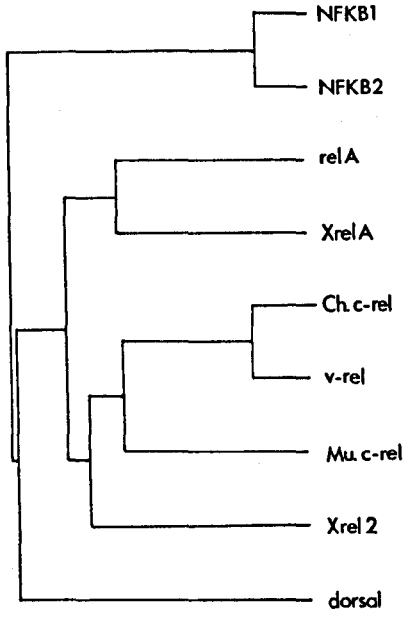
[1] Note the interaction of IκBα with p52/relB is much weaker than that with p50/relB (Dobrznaski et al., 1994).

FIG. 1:- A. Table of nomenclature for the rel family.

Gene	Product(s)
RelA	p65, p65Δ
RelB	relB
c-*rel*	Rel
NFKB1	p105, p50 (p105 cleavage product), IκBγ, IκBγ-1 and IκBγ-2 (alternative splicing products)
NFKB2	p100, P52 (p100 cleavage product)
v-*rel*	v-Rel
XrelA	XrelA
Xrel.2	Xrel.2
dorsal	dorsal

The first five of these genes have homologues which have been cloned in humans, mice and chicks. It is these genes which are concentrated on for most of this section (1.1) of this section v-rel is a viral oncogene from avian reticulovirus. The *Xenopus* members of the rel family, *XrelA* and *Xrel.1* are dealt with in a separate section (1.1.7.). *Dorsal* is a *Drosophila* member of the rel family member. Its developmental function is disscussed in detail in section 1.2.

B. Sequence analysis of the rel family.



The above figure shows relationships between sequences of members of the rel family, reproduced from a pileup sequence comparison on GCG.

The C-terminal inhibitory domains of NFKB1 and NFKB2 mentioned previously are actually quite closely related in sequence to members of the IκB family, and in fact differential splicing of the NFKB1 transcript produces an IκB protein in its own right, known as IκBγ. IκBγ protein is capable of inhibiting nuclear localisation of homodimers of p50 and Rel, and p50/p65 heterodimers[2] (Grumont and Gerondakis, 1994). It has recently been shown that the NFKB1 gene has two further products resulting from alternative splicing (Grumont and Gerondakis, 1994). These products, known as IκBγ-1 and IκBγ-2, are smaller isoforms of IκBγ which lack IκBγ's putative Protein kinase A (PKA) phosphorylation site, suggesting their activity may be controlled differently. IκBγ-1 and -2 are also more limited in their specificity for rel dimers, only inhibiting DNA bindng by p50 containing dimers. Unlike other IκB proteins with inhibitory functions, IκBγ-2 is found predominantly in the nucleus.

A further member of the IκB family has a completely different function. Bcl3 operates in the nucleus, where it binds to p50 and p52 homodimers in a phosphorylation dependent manner (Nolan *et al.*, 1993). Bcl3 has transactivating activity which becomes functional when Bcl3 is bound to p50[3] or p52 homodimers (which alone are not transactivating) bound to a relevant site (Bours *et al.*, 1993; Fujita *et al.*, 1994). Bcl3 does not bind to p65 homodimers, or to NF-κB (p65/p50) (Nolan *et al.*, 1993).

1.1.2. Activation of NF-κB

The rel family dimer whose mechanism of activation has been most thoroughly investigated is the dimer of p50 and p65, known as NF-κB. NF-κB is activated by a wide range of factors, but most is known about activation by the cytokines IL-1 and TNF-α . Evidence has gradually been accumulating that ceramide produced as a result of signal-dependent hydrolysis of sphingomyelin, acts as a second messenger involved in a pathway leading to activation of NF-κB. Levels of endogenous ceramide are rapidly stimulated, concommitant with a rapid decrease in sphingomyelin levels, in both TNF-α and IL-1β treated cells (Schutze et al., 1992; Mathias et al., 1993. A possible mechanism for the action of ceramide as a second

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[2] Interaction of IκBγ with $(c\text{-}rel)_2$ and p50/p65 is still controversial, and contrary reports exist (e.g.:- Nolan et al., 1993).

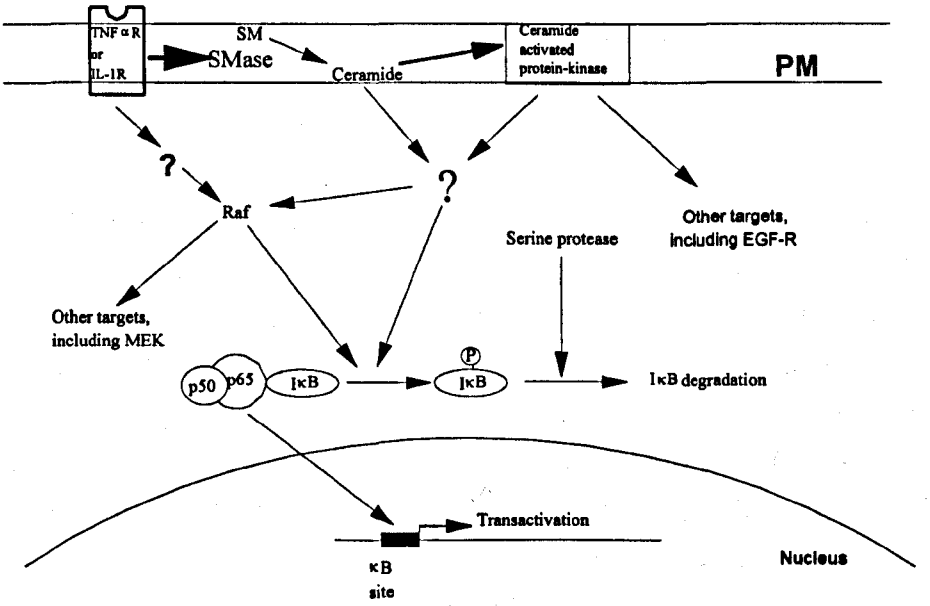
[3] Reports of an inhibitory function for Bcl3 acting against p50 homodimers (e.g.:- Franzoso et al., 1992) are probably an artefact due to the use of a deletion clone of NFKB1 which has a product longer than mature p50 .

messenger is suggested by the isolation of a ceramide activated kinase (Mathias *et al.*, 1991). Introduction of a cell permeable analogue of ceramide into Jurkat T-cells activates NF-κB in a dose-dependent manner (Schutze *et al.*,1992). NF-κB can also be activated by both ceramide and sphingomyelinase in cell free extracts of both Jurkat T-cells and a human monocyte line (U937) (Machleidt *et al.*, 1994). This activation can be inhibited using a range of serine protease inhibitors. These same protease inhibitors have been shown to prevent IκB dissociation and degradation in response to a range of NF-κB activating stimuli (Pallombella *et al.*, 1994). Two possible scenarios could account for this data. Either ceramide is, directly or indirectly, activating a serine type protease, which subsequently degrades IκB, or ceramide acts to cause the modification (possibly phosphorylation) of IκB, which somehow marks it out for degradation. The latter model would fit in with the evidence for IκB phosphorylation being necessary for NF-κB activation, and with the observation that the range of protease inhibitors able to inhibit IκB degradation corresponds to those able to inhibit the proteasome (Machleidt *et al.*, 1994).

The intracellular tyrosine kinase raf has also been implicated in NF-κB activation by TNF-α and other activators. Finco and Baldwin have shown recently that a dominant negative form of raf-1 (which has an inactivated kinase domain, and is thought to act by saturating the binding sites of one or more upstream activators) can block activation of NF-κB by TNF-α, serum and phorbol esters when overexpressed in Balb/c 3T3 cells (Finco and Baldwin, 1993). They also demonstrated that overexpression of activated forms of raf-1 and ras (an upstream activator of raf-1) was sufficient for NF-κB activation in the same cell line. Two lines of evidence suggest that raf-1 may activate NF-κB by acting directly on IκB. Firstly, recombinant raf-1 can phosphorylate recombinant, purified IκB *in vitro*, and secondly raf-1 and IκB have been shown to associate *in vivo* using the yeast two hybrid system (Li and Sedivy, 1993). No work appears to have been carried out so far on the relationship of ceramide and raf-1 in this pathway (e.g.:- does dominant negative raf block ceramide activation of NF-κB). However, assuming the interaction of raf-1 and IκB are physiologically relevant, one might expect raf-1 to act downstream of, or in parallel with the ceramide pathway of activation (see figure 2)

It has recently become apparent that proteolytic processing of p105 is , at least in some contexts, a signal-dependent process, which can by stimulated by, for example, TNFα and PMA (Mellitis et al., 1993; Mercurio et al., 1993). Like the degradation of IκBα this process involves phosphorylation prior to proteolytic degradation which again appears to involve the proteasome (Pallombella *et al.*, 1994).

FIG. 2:- Model for activation of NF-κB by TNFα / IL-1



Abbreviations:-
SM = sphingomyelin; SMase = Sphingomyelinase; EGF-R = epidermal growth factor receptor
PM = Plasma Membrane
P(circled) = phosphate.

1.1.3. Variation in DNA binding specificity between different rel dimers.

The rel family were initially characterised as binding to a group of decameric sequences known as κB sites which are variations on a palindromic site with the sequence 'GGGAATTCCC' in which the first 4 nucleotides, and the terminal 'C' are always conserved. A range of κB sites of this form from various promoters are summarised in figure 3 . Even within this limited range of sites there is difference in specificity between different rel dimers (see figure 4). However, it is increasingly becoming apparent that the range of sites bound by rel dimers is much larger than the these canonical κB sites. A study by Kunsch and colleagues using a PCR selection assay to define the complete range of sites bound by homodimers of p65, p50, and Rel has highlighted a range of new sites not so far found in promoters (Kunsch *et al.*, 1992). This study also defined sites specific for each of the three homodimers which could be a useful tool for investigating the presence of particular homodimers, and have already proved useful for studying which residues confer specificity (see page 12). The sites found to bind Rel homodimers included some of the sites most

divergent from the palindromic κB site (e.g.:- CTAAAAAACC), and recently c-rel has been shown to bind to an important site in an intron of the IFN-γ gene which is also almost totally unlike a 'canonical' κB site (AATTTTCC).

1.1.4. Transactivation by rel family members

In naturally occurring promoters the context of a site is frequently an important factor in whether transactivation occurs. For example, transactivation by NF-κB from an HIV-LTR driven reporter is dependent on the presence of Sp1 sites adjacent to the two κB sites (Perkins *et al.*, 1992). Despite this it has become common to test rel dimers for the ability to transactivate from artificial promoters consisting of a number of adjacent copies of particular κB site next to a minimal promoter (e.g.:- the thymidine kinase promoter). This method is not, of course, able to distinguish between dimers which can transactivate alone, and those requiring further interactions with factors present in vivo (e.g.:- $(p50)_2$ or $(p52)_2$ requirement for Bcl3). Data from such experiments is summarised in figure 4.

FIG. 3:- Types of κB site.

κB site	Sequence
Ig (κ-light chain) and HIV LTR	GGGACTTTCC
IL-2	GGGATTTCAC
IL-2R	GGGAATCTCC
Il-6	GGGATTTTCC
Urokinase	GGGAAAGTAC
H2	GGGATTCCCC
IFN-β	GGGAAATTCC
Palindrome (PD)	GGGAATTCCC

FIG. 4:- Table of rel dimers and their activities.

Dimers	Characterised IκB family interactions[1]	Binding sites[2]	Transactivation[3]
p50/p65 (NF-κB)	IκBα, IκBβ, IκBγ, IκBγ-1, IκBγ-2 (nuclear (Not Bcl3)	Ig/HIV, IFN-β, H2, IL-2, IL-2R, IL-6, Urokinase	IgG/HIV, IFN-β (requires HMG I(Y), H2
p52/p65	IκBα	Ig/HIV, PD, H2, IL-2R	Ig/HIV (but not H2, IL-2R or PD)
p50/relB	IκBα	Ig/HIV, PD	Ig/HIV, PD
p52/relB	IκBα (Weakly binding)	Ig/HIV, PD	
p50/C-rel	IκBα	Ig/HIV	
p52/C-rel	IκBα	IL-2R	
p65/C-rel	IκBα	Urokinase (Not Ig/HIV)	
$(p50)_2$	IκBγ, IκBγ-1, IκBγ-2 (nuclear, inhibitory), Bcl3 (transactivating) (Not IκBα or β)	Ig/HIV, IL-2, IL-2R, IFN-β, H2, but not IFN-γ intron.	H2, Ig/HIV (but not IFN-β)
$(p52)_2$	Bcl3 (transactivating)	H2 (but not Ig/HIV)	
$(p65)_2$	IκBα, IκBβ, IκBγ (Not IκBγ-1 or -2 or Bcl3)	Ig/HIV, IFN-β, IL-2R	IL-2R (repressor for IL-6)
$(C\text{-}rel)_2$	IκBα, IκBβ, IκBγ (Not IκBγ-1 or -2)	IL-2R, IL-6, IFN-γ intron (but not Ig/HIV, or IL-2)	

Adapted and updated from Liou & Baltimore, 1993, references for DNA binding specificity and transactivation can be found there. References for IκB specificty can be found in the text (see section 1.1). It is important to note that not all of potential interactions / activities in these categories have been characterised. Where there is known to be no interaction this is also mentioned.
Note RelB does not homodimerise, and heterodimerises only with p50 and p52 (Bours et al., 1994).

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[1] Note, it is still not clear whether interaction with a particular rel subunit allows interaction with all dimers containing that subunit. I have therfore only included characterised interactions with particular dimers.

[2] All results are as tested by GMSA (see section 4.1 for details). The list only includes binding sites characterised in promoters, and does not take into account additional binding activities characterised by PCR selection.

[3] Transactivation as measured from artificial promoters consisting of multiple adjacent κB sites and a minimal promoter.

1.1.5. Physiological roles of rel family members.

There has long been circumstantial evidence for a role for the rel family in the functioning and development of the vertebrate immune system. Many of the genes containing promoter elements characterised as being responsive to activation by rel family dimers, are involved in the immune acute phase and inflammatory responses (Liou and Baltimore, 1993). Where expression patterns of rel family members are restricted they also suggest a role in the immune system. The expression of c-rel, for example, is restricted to the hematopoietic organs in the mouse, with highest levels in B and T lymphoid cells (Grumont and Gerondakis, 1990). c-*rel* expression in the B cell lineage during maturation is a good illustration of the potential importance of combinatorial variation of rel dimers in differentiation. c-*rel* expression is stage-specific so that, in pre-B cells the main rel dimer is p50/p65, but in mature B cells it is p50/Rel (Grumont and Gerondakis, 1994). *RelB* expression also suggests a role in the immune system, being localised to the thymus and lymph nodes. *rel A* and *NFKB1*, on the other hand, are thought to be ubiquitously expressed, so roles outside of the immune system are possible.

Recently homozygous null alleles of a number of members of the rel family have been generated in mice by homologous recombination. It is important to note with such data that as there appears to be significant overlap in the activities of some rel dimers it is possible that in some situations other rel family members may be able to substitute for an eliminated rel protein. Investigation of this possibility will have to await the generation of double mutant mice. So far, none of the homozygous null phenotypes have included developmental defects. Embryos which are homozygous null for NFKB1 develop normally, but have a variety of immune response defects as adults (Sha *et al.*, 1995). In support of the redundancy hypothesis many genes characterised as possible targets for p50 containing dimers are expressed normally in these mice. RelB -/- mice also develop relatively normally apart from having a number of hematopoietic defects, and a reduced population of dentritic cells in the thymus (Weih *et al.*, 1995). These mice also have impaired cellular immunity and infiltration of a number of organs by inflammatory cells leading to inflammation. Embryos homozygous null for *relA* again have no obvious developmental abnormalities. The major defect observed in mice which lack *relA* is that the liver undergoes apoptosis at about 15 dpc, after apparently normal prior development (Beg *et al.*, 1995). Finally, mice lacking c-rel also have immune defects, most or all of which may result from the inability of mature B and T cells from these mice to transduce mitogenic signals (Kontgen *et al.*, 1995).

1.1.6. Mapping the domains of activity of rel family members.

Extensive deletion and *in vitro* mutagenesis analysis of *NFKB1*, *relA* and v-*rel* have lead to the mapping of regions essential for DNA binding and dimerisation (see figure 25 for schematic representaions of these domains). The first attempts at domain mapping were crude deletions of *NFKB1*, which lead to the identification of a region at the N-terminal end of the RHD, necesssary for DNA binding. For example, the deletion p50ΔSP which lacks residues 10-201 (along with the IκBγ encoding domain) encodes a protein which lacks DNA binding activity, but is still able to dimerise with wild type rel family members (Kieran *et al.*, 1990). The region which has been deleted in p50ΔSP contains a 15 residue stretch which is highly conserved in all the known members of the rel family (see figure 5A). Analysis of the functions of individual residues within this region has been carried out by *in vitro* mutagenesis (summarised in figure 5B). As well as identifying residues essential for DNA binding, or necessary for binding with normal affinity, one residue has been shown to be an important determinant of sequence specificity (see figure 5B). Mutagenesis of this residue in relA to its NFKB1 equivalent causes a broadening of specificity to include targets of NFKB1, whereas mutagenesis of the residue in NFKB1 to its relA equivalent is sufficient to change its specificty to that of relA as tested using probes specific for each homodimer (Coleman *et al.*, 1993). The recently published structure of $(p50)_2$ bound to a consensus κB site (GGGGAATCCC) supports a role for this region in determining DNA binding specificity. Residues 56-71(see figure 5A) form a 'recognition loop' that projects into the major groove of the bound DNA and interacts directly with bases in the site (Ghosh *et al.*, 1995; Müller *et al.*, 1995).

Perhaps because the relevant domain(s) is/are not as well conserved or localised, mapping of regions and residues necessary for dimerisation has been less successful. One of the first clues to the position of residues essential for dimerisation was the isolation of a naturally occuring alternative splice form of relA known as p65Δ , which lacks amino acids 222 to 231 (Narayanan *et al.*, 1992). This protein is unable to dimerise, stabilise IκB, bind DNA or transactivate (the loss of the latter two functions are likely to be a secondary consequence of loss of dimerisation activity). Interestingly, despite the loss of all these activities, this deletion has transforming activity, although the mechanism of this activity remains obscure. The equivalent region of p50 has also been shown to be essential for dimerisation (Bressler *et al.*, 1993). However, residues outside this region also have a role in dimerisation. Mutagenesis of a highly conserved cysteine residue (C216 in rel A), and of residues

FIG. 5:- (a) Comparison of the highly conserved regions of rel family DNA binding domains

```
Dorsal (58-72)      K F L R F R Y E C E G R S A G
hI-rel (131-145)    R G M P F R Y E C E G R S A G
mRelB (115-129)     R G M R F R Y E C E G R S A G
hNFKB1 (54-68)      R G F R F R Y V C E G P S H G
hNFKB2 (45-59)      R G F R F R Y G C E G P S H G
v-Rel (27-41)       R G T R F R Y K C E G R S A G
mc-Rel (19-33)      R G M R F R Y K C E G R S A G
hRelA (30-44)       R G M R F R Y K C E G R S A G
XrelA (30-44)       R G M R F R Y K C E G R S A G
Xrel.2 (33-47)      R G M R Y R Y K C E G R C A G
                              *                 *
                    - - - - - # # - # # # - - - #
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Completely conserved residues are marked with a '#'. Residues which differ between Xrel.2 and all other rel family members are marked with a '*'. No published data currently exists on the effects of mutagenesis of these residues. Gene name prefixes: 'm' denotes mouse and 'h' denotes human

(b) Summary of specificity and affinity changes due to mutagenesis of single amino acids in this region.

(Note:- Specificity differences can be tested using specific probes isolated by Kunsch and colleagues (see page 12)):-

GENE	SUBSTITUTION	EFFECT ON SPECIFICITY/AFFINITY
NFKB1	H67R	p50 specificity to p65 specificity
RelA	A43H	p50 and p65 specificity
RelA	R41P	p50 and p65 specificity
RelA	M32F	Decreased affinity, same specificity
RelA	K37V	Decreased affinity, same specificty
v-*rel*	C35S	Binding affinity increased[4]
v-*rel*	R27W	Binding abolished
v-*rel*	R30Q	Binding abolished
v-*rel*	R32N	Binding abolished

(from Kumar *et al.*, 1992 and Coleman *et al.*, 1993)

[4] This residue appears to be involved in redox control of v-rel activity, as unlike the wild-type the binding affinity of the C35S mutant of v-rel is not increased in reducing conditions (Kumar et al., 1992).

in a conserved consensus PKA binding site at 271-274 are able to abolish the ability of p65 to homodimerise, but not to heterodimerise with p50 (Ganchi *et al.*, 1993).

1.1.7. Rel family members in *Xenopus*.

The aim of this section is to outline the results of work on XrelA prior to the start of this project. More recent results are detailed, where appropriate, in later chapters.

XrelA: Cloning, properties and expression.

A *Xenopus* member of the rel family, now known as XrelA, was isolated by Jill Richardson (Richardson, 1991; Richardson et al., 1994), during a screen for *Xenopus* rel genes using a fragment of the v-*rel* RHD to screen an oocyte library. cDNAs encoding two slightly different RNAs were isolated by this method, called XrelA.1 and .2., and found to be identical (except at two residues) to the clone Xrel.1, independently reported by Kao and Hopwood (Kao and Hopwood, 1991). XrelA.2 was used throughout this study and will henceforth be referred to as XrelA. XrelA is most similar at the amino acid level to the relA subunit of NF-κB (see figure 1B) having a similarity of 77.5% to relA in the RHD (allowing for conservative changes). XrelA and relA are identical over most of the region of the RHD which has been shown to be necessary for DNA-binding activity (see section 1.1.6), and is absolutely identical in the part of this region characterised as controlling specificity (see figure 5). It seems likely, therefore, that the DNA-binding specificity of XrelA, and relA will be very similar. As can be seen from figure 6, XrelA is also very similar to relA over the regions identified as essential for homo- and heterodimerisation. XrelA also contains the conserved nuclear localisation signal (KRKR; residues 300-304) found in all other rel family members. The C-terminal domain of XrelA has a much lower degree of similarity with relA (only 27 %). By analogy with relA this region would be expected to have a transactivating function. The C-terminal domain of relA has been classed as an acidic-type activation domain. Recent work has characterised short sequences in this domain known as acidic activation modules, which have been shown to be capable of acting independently as transactivators in HeLa cells when fused to the DNA binding domain of yeast transcription factor Gal-4 (Blair *et al.*, 1994). Similar putatively conserved sequences can be found in the C-terminal domain of XrelA, as shown in figure 7.

FIG. 6:- Comparison of rel family dimerisation domains

```
mc-rel  (205)   FLLCDKVQKD DIEVRFVLND .....WEARG
Xrel.2  (218)   FLLCDKVQKD DIEVRFFTDN .....WEAKG
mrela   (213)   FLLCDKVQKE DIEVYFTGPG .....WEARG
Xrela   (213)   FLLCDKVQKE DIEVIFGLGN .....WEARG
hNFKB1  (270)   YLLCDKVQKD DIQIRFYEEE ENGGVWEGFG
                ********  **   *          **  *

mc-rel  (259)   VKMQLRRPSD
Xrel.2  (273)   VKMQLRRPSD
mrela   (268)   VSMQLRRPSD
xrela   (266)   VQMQLRRPSD
hNFKB1  (329)   VFVQLRRKSD
                *  **** **
```

Sequence alignments taken directly from complete alignments using Pileup. All residues whose function has been investigated by deletions or by *in vitro* mutagenesis (detailed in table below) are shown in bold.Gene name prefixes:- m = mouse, h = human.

GENE	Substitution/deletion	Effect	Reference
relA	222-231 (naturally occuring deletion, relAΔ)	Abolishes all dimerisation	Narayanan *et al.*, 1992
h*NFKB1*	269-278 (equivalent deletion to relAΔ)	Abolishes all dimerisation	Bressler *et al.*, 1993
m*rela*	C216S	Abolishes homodimerisation, but not heterodimerisation.	Ganchi *et al.*, 1993
m*rela*	S276A (part of conserved PKA consensus site (RRPS)	Abolishes homodimerisation, but not heterodimerisation.	Ganchi *et al.*, 1993

That XrelA has transactivation activity has been confirmed by experiments demonstrating that XrelA can stimulate transcription from an HIV-LTR driven CAT reporter construct (pLC2R; Herbomel *et al.*, 1984), when over-expressed in embryos (Richardson *et al.*, 1994). However, transactivation is not seen in this assay with very high levels of XrelA expression (2 ng/embryo). Interestingly, at such high levels of expression transactivation from a reporter driven by the thymidine kinase promoter (Edlund *et al.*, 1985), which does not contain a κB site, is decreased. This could be explained by XrelA causing a general inhibition of activated transcription, known as squelching. Squelching is thought to occur when an overexpressed transcription factor sequesters a protein or protein(s), via interaction with its transactivation domain, which are required generally for activated, but not basal, transcription. It has been shown recently that acidic activation modules from the relA transactivation domain have squelching activity when fused to the DNA binding region of Gal4 and overexpressed in HeLa cells, as tested by inhibition of transactivation by the acidic class transactivation domain of VP16 (Blair *et al.*, 1994). As with other examples of squelching, basal transcription was unaffected, as shown by the failure of overexpressed relA to inhibit transactivation by a GAL4-Tat fusion.

To test for a general decrease in transcription due to squelching in embryos expressing high levels of XrelA, the incorporation of tritiated uridine into poly $(A)^+$ RNA as a proportion of incorporation into total RNA was measured (Richardson *et al.*, 1995). No significant reduction in this ratio was seen in embryos over-expressing XrelA compared to controls. However, as this method is fairly crude it may not be useful in detecting general decreases in mRNA transcription rates which are nevertheless significant, especially when one considers that basal transcription is unaffected by squelching effects.

The spatial and temporal expression pattern of XrelA has been analysed by Northern blotting. XrelA is expressed at low levels maternally, but levels increase sharply after MBT, rising to highest levels in the late blastula / early gastrula, then tailing off by neurula stages, although transcripts can still be detected at stage 35 (Richardson *et al.*, 1994). No obvious dorsal-ventral distribution of transcripts was observed in the early embryo.

FIG. 7:- Alignments of putative conserved acidic activation modules

```
                535-545 (muRelA)

muRelA      S I A D M D F S A L L
huRelA      S I A D M D F S A L L
XRelA       S L F E L D F S S L L
            *         * * *   * *
```

and more tentatively:-

```
                442-453 (muRelA)

muRelA      L Q F - - - D D E D L G A L L
huRelA      L Q F - - D A D E D L G A L L
 XRelA      P S F P G D A N L D L V E M L
               *             * *       *
```

Alignments taken directly from full sequence alignments (Richardson, 1991) using Clustal analysis (programs 1,2 and 3; Higgins and Sharp, 1988)
Note that the central 'conserved' phenylalanine residues in each case have been shown to be essential for the function of these modules (Blair *et al.*, 1994).

Phenotypes due to injection of RNA encoding XrelA, and RNA encoding a transactivation deficient deletion of XrelA

A two-pronged approach has been used to investigate the developmental role of XrelA. The phenotypic effects of over-expressing XrelA have been investigated in some detail (Richardson *et al.*, 1995). This approach has proved successful in revealing developmental activities of a number of genes expressed early in *Xenopus* development. The homeobox gene *goosecoid* (*gsc*) for example, has the ability to induce secondary axis formation when injected as RNA into ventral blastomeres (Cho *et al.*, 1992; also see section 7.1.3.). However, it should be noted that one potential complication of this approach in the case of XrelA is that overexpression would lead to the expression of homodimers, which may or may not be the predominant species of dimer present in the embryo.

High level overexpression of *XrelA* in embryos (>2 ng) resulted in arrest of embryonic development at stage 10 (early gastrula), just after formation of the dorsal blastopore lip. As this phenotype is seen mainly at or above the same levels of expression of XrelA as 'squelching' of transcription driven by the thymidine kinase promoter, it seems likely to be a consequence of squelching. In fact it is similar to the phenotype caused by treatment of embryos with the transcriptional inhibitor α-amanitin, which also blocks gastrulation, although in this case prior to the formation of the dorsal blastopore lip (Newport and Kirschner, 1982). The squelching hypothesis is also supported by an analysis of early marker expression using RNase protection assays (Richardson *et al.*, 1995). The results of these experiments show a decrease in the levels of transcripts of all markers tested (*Xbra, goosecoid, pintallavis, Xwnt-8, Xsna* and *noggin*) in embryos injected with high levels of XrelA (>2 ng/embryo) when compared to the levels of RNA of the maternal and zygotic housekeeping gene ornithine decarboxylase (Richardson *et al.*, 1995).

Embryos injected with lower levels of XrelA complete gastrulation and often develop with a disruption of the mid-dorsal axis. Somite segmentation and organisation is affected and the nervous system is small and poorly organised, in contrast, notochord seems to develop normally. In addition, thickening of the epidermis into multi-layered patches is often seen in these embryos. The least affected embryos exhibit a mid axis 'kink' associated with local disruption of axial tissue. The difficulty with interpreting this phenotypic data, compared say to the phenotype resulting from ventral injection of *gsc* RNA, is that it cannot be fitted easily into currently accepted models of *Xenopus* early development (see section 1.3).

The other approach which has been used to investigate the developmental function of XrelA is the use of dominant inhibitory deletions[5] of XrelA to study the phenotypic effects of eliminating the activity of XrelA in the embryo. The only such deletion clone whose activity and phenotypic effects have been characterised is one lacking the C-terminal transactivation domain. This deletion clone, known as XrelAΔ 222 (the C-terminal 222 amino acids are deleted), can inhibit both background and XrelA stimulated levels of transcription from pLC2R (Richardson *et al.*, 1994; Fig. 29). This probably occurs by the saturation of promoter sites by homodimers of XrelAΔ222. It is also possible that XrelAΔ222 could act by forming transactivation deficient heterodimers with XrelA, although this is difficult to prove. Dominant

[5] There is a general discussion on the design of dominant inhibitory deletions of rel family members in chapter 5 (sections 5.1 and 5.2).

negative transcription factors which act by saturating promoter sites have the disadvantage that, when expressed at highly non-physiological concentrations they may interact with promoter elements which are not normal targets of the factor to be inhibited. This is not a problem for which controls may be easily designed.

Embryos injected with RNA encoding XrelAΔ222 exhibit a markedly different phenotype to those over-expressing wild-type XrelA. They appear to gastrulate 'over-vigorously', at least posteriorly, so that the resulting blastopore ends up as a pit. At later stages this develops into a large cloaca, sometimes encompassing the whole of the posterior end of the trunk, which tends to be shortened. In more extreme embryos tail extension is inhibited, and somewhat less frequently, head defects are seen. In extreme cases the head is completely lost. Histological examination of these embryos revealed that they had relatively normal mid-trunk regions, but were increasingly disorganised towards the posterior, particularly with respect to segmentation of the somites.

Xrel.2: A member of the rel family with similarities to c-rel

Recently, a second *Xenopus* rel homologue has been identified with significant homology to c-*rel* (David Tannahill, pers. comm.). Like XrelA, it is expressed both maternally and zygotically and so is a potential heterodimerisation partner of XrelA.

Close comparison of the sequence of Xrel.2 with other rel family members in the region of the rel homology domain (RHD) shown to be necessary for DNA-binding activity, and implicated in controlling DNA-binding specificity (see section 1.1.6) suggests that the similarity of *Xrel.2* and c-*rel* could be misleading with respect to DNA binding-specificty. *Xrel.2* has substitutions (albeit relatively conservative ones) at two residues in this region which are completely conserved in all other rel family members (see figure 5A, page 13). The substituted phenylalanine (which becomes a tyrosine residue in Xrel.2) lies between two conserved arginines which in p50 form bidentate hydrogen bonds with guanines 3 and 4 in the consensus site (Ghosh *et al.*, 1995). The other substituted residue, a cysteine instead of the serine residue found in other rel family members, is adjacent to a residue essential for conferring specificity differences between relA and NFKB1 (see Fig. 5 AandB, page 13). All of this points to the possibility of Xrel.2 having novel DNA binding specificity properties.

1.2. D-V Patterning in *Drosophila* - An example of pattern specification by a rel family member.

The aim of this section is to outline the role of the *Drosophila* rel family member *dorsal* in dorsal - ventral (D-V) patterning of the *Drosophila* embryo as an illustration of the potential of rel family members for pattern specification. The first section deals with the mechanisms by which a gradient of nuclear concentration of dorsal from ventral to lateral is specified during the syncytial blastoderm stage. This is reviewed in much more detail elsewhere (e.g.:- Chasan and Anderson, 1993). The second section deals with the mechanisms by which target gene expression is regulated by the nuclear concentration of *dorsal* protein leading ultimately to the specification of D-V pattern.

1.2.1. Control of dorsal localisation

During *Drosophila* oogenesis one germ cell divides four times to produce 16 cells, which remain connected by cytoplasmic bridges. One of these cells becomes the oocyte, while the rest become nurse cells which export large amounts of protein and RNA into the oocyte. Surrounding most of the oocyte are somatic cells known as ovarian follicle cells. These cells are responsible for secreting the vitelline membrane and the chorion (eggshell). The chorion becomes visibly polarised both antero-posteriorly and dorso-ventrally during oogenesis. Fertilisation is followed by rapid synchronous nuclear division which occurs without cellular cleavage. After nine divisions most of the nuclei migrate to the periphery. This is called the syncytial blastoderm stage, and it is not until a further four divisions have taken place that cellular cleavage occurs.

Genetic analysis has identified 18 maternally expressed genes required to define D-V pattern in the *Drosophila* embryo (see figure 8). Six of these genes are required for both embryo and chorion patterning (the future polarity of the embryo can be predicted by the D-V asymmetry of the chorion). The remaining 12 genes are required for patterning of the embryo after fertilisation. Embryos from flies which are homozygous for null alleles of eleven of these genes (known as the dorsal group) produce completely dorsalised embryos, whereas null alleles of the remaining gene (*cactus*) give a completely ventralised phenotype. Isolation of partial loss of function, weakly dorsalising mutants of the dorsal group originally lead to the proposal that

FIG. 8:- Table of maternal effect genes controlling D-V patterning.

	Gene	Loss of function phenotype	Protein encoded
Eggshell and embryonic patterning	*fs(1)K10*	dorsalising	bHLH protein
	cappuccino	dorsalising	?
	spire	dorsalising	?
	torpedo	ventralising	EGF-R homologue
	gurken	ventralising	?
	cornichon	ventralising	?
Embryonic patterning	*pipe*		?
	nudel		?
	windbeutel		?
	gastrulation defective	Dorsal group genes	(serine protease ?)
	snake		serine protease
	easter		serine protease
	spätzle		secreted protein
	Toll		transmembrane protein
	pelle		Raf/Mos type tyrosine kinase
	tube		(no known homologues)
	dorsal		Rel family member
	cactus	ventralising	IκB family member

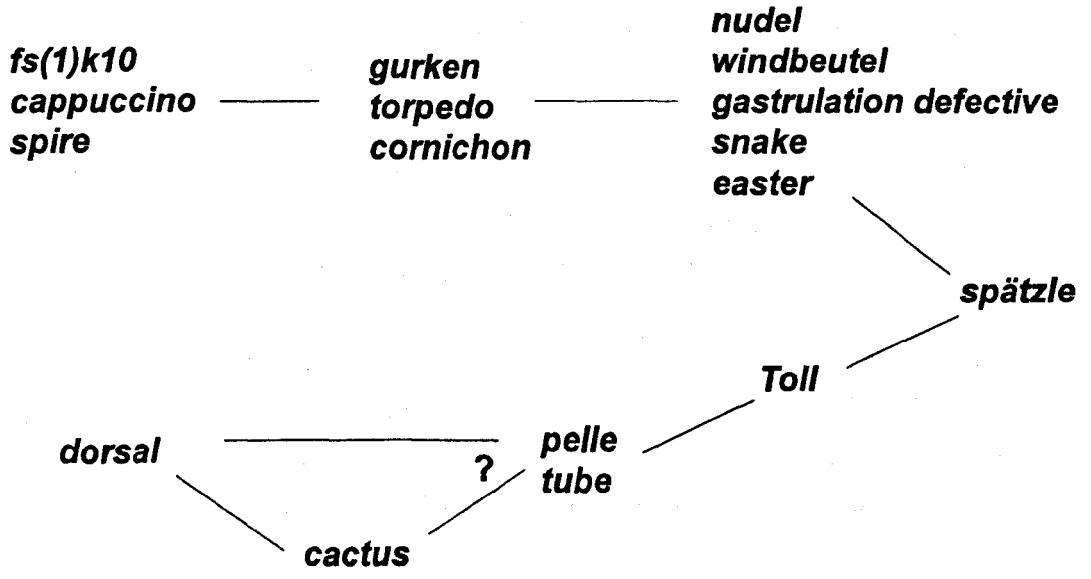
Adapted from Chasan and Anderson, 1993.

D-V patterning relied on a gradient of a ventralising signal, the absence of which leads, by default, to dorsalisation. The weakly dorsalising (lateralising) mutants of the dorsal group could then be interpreted as depleting this ventralising signal. The proteins encoded by these genes are now known to be involved in a chain of events which carries a signal from the follicle cells surrounding the embryo to peripheral nuclei during cleavage of the syncytial blastoderm. This ultimately leads to the formation of a gradient of nuclear localisation of dorsal protein from ventral (high) to lateral (none) across the embryo, between cleavage cycles 10 and 14.

It is possible to genetically dissect the order in which a group of genes act in a linear pathways, such as signal transduction pathways, by using double mutant

analysis. This makes use of the fact that embryos carrying two mutant genes involved in such a pathway should display the phenotype of the further downstream of the two genes. The phenotype due to mutation in the downstream of the two genes is referred to as being epistatic to the phenotype due to the other mutation. This technique is obviously only applicable where the mutations in question involve loss of function, or are dominant alleles, and give different phenotypes. Epistasis analysis has therefore allowed the positioning of maternal D-V patterning genes with respect to *cactus*, *torpedo* and *gurken* (ventralising as null alleles) and *Toll*, *easter* (ventralising as dominant alleles) and *spätzle* (lateralising as a dominant allele) (see figure 9).

FIG. 9:- Order of genes in D-V pathway by genetic dissection.



Updated from Chasan and Anderson, 1993.
Note:- It is unclear from the epistasis experiments whether *tube* and/or *pelle* acts directly on *dorsal*, *cactus* or both.

This model correlates well with data on the localisation of gene products. Pipe, windbeutel and nudel are all required in the follicle cells (Govind and Steward, 1991), where they are postulated to encode proteins responsible for laying down the initial asymmetric patterning signal. Snake, easter and spätzle are all present in the perevitelline space and in fact, all three of these mutant phenotypes can be rescued by injection of perivitelline fluid from a WT host into the perivitelline space of a mutant donor. Toll is present in the plasma membrane, and tube, pelle and cactus are present in the embryo itself.

snake and *easter* both encode trypsin-like serine proteases which are secreted as inactive zymogens requiring protease cleavage for activation (Delotto and Spierer, 1986; Chasan and Anderson, 1989). The cloning and characterisation of these genes led to the suggestion that they might participate in a self amplifying zymogen cascade, similar to that involved in blood clotting or complement activation (Chasen *et al.*, 1992). This hypothesis fits with the observation that the D-V orientation of mutant embryos rescued by injection of wild-type perivitelline fluid corresponds to that of the chorion rather than depending on the position of injection. This form of rescue experiment provided the basis for a form of epistasis experiment for *snake* and *easter*. These experiments involved injection into embryos of RNA encoding the catalytic regions of *snake* and *easter* fused to a signal sequence to direct secretion into the periviteline space. In wild-type embryos these RNA's produce a dominant lateralised or ventralised phenotype which is epistatic with respect to null alleles of *nudel*, *pipe*, *winbeutel* and *gastrulation defective* (Smith and Delotto, 1994; Chasan *et al.*, 1992). These experiments also place *easter* downstream of *snake*, but upstream of *spätzle* and *Toll*. Recently, a dominant gain of function allele of *spätzle* has been isolated which produces a completely lateralised phenotype which is epistatic to *easter*. Unlike *snake* and *easter*, increasing the amount of wild type protein in the periviteline space by RNA injection into embryos does affect the phenotype.

These experiments support a model in which the *easter* gene product acts downstream of *snake* to activate *spätzle*. In this model *easter* and *snake* encode elements in a zymogen cascade, in which *snake* is activated by an initial asymmetric cue acting through, or in parallel with, the product of *gastrulation defective*.

Toll itself encodes a transmembrane protein which shows weak homology to the Interleukin 1 receptor in its intracellular domain. *In vitro* mutagenesis of residues conserved in the intracellular domain of the IL-1 receptors of humans, chickens, and mice has identified a number a functionally essential residues. The functional significance of two equivalent residues present in *Toll* is indicated by the finding that they are substituted in two recessive dorsalising mutants of *Toll*. As genetic and biochemical evidence suggest that *spätzle* acts immediately upstream of *Toll* it seems likely that *spätzle* encodes the *Toll* ligand.

Transduction of the signal from Toll which leads to nuclear localisation of dorsal is still not well understood. *Tube* has no known homologues, but is known to be localised evenly to the plasma membrane in syncytial blastoderm embryos (Galindo *et al.*, 1995) and *pelle* is predicted by sequence similarity be a raf/mos type ser/thr

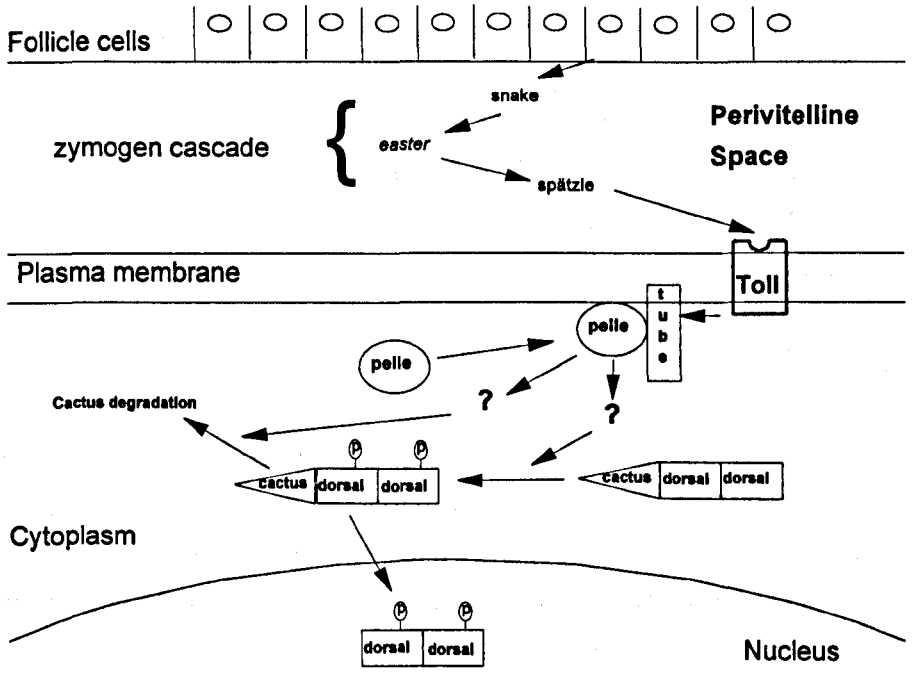
kinase (Shelton and Wasserman, 1993). Recently pelle and tube have been shown to associate physically independently of any activating signal, using the yeast two hybrid system (Galindo *et al.*, 1995). This interaction appears to be essential, as two loss of function tube mutants do not interact with pelle in this assay. Intriguingly, a dominant gain of function *pelle* can be created by fusing the kinase domain of *pelle* to a non-functional mutant of the transmembrane receptor *torso*, suggesting that activation of pelle simply requires localisation to the plasma membrane. This is analogous to the activation of raf by ras, where ras is thought to be involved in recruiting raf to the plasma membrane where it can be activated by phosphorylation (Marais *et al.*, 1995). This dominant gain of function *pelle* has been used in epistasis experiments to show that pelle functions downstream of tube. One possible interpretation of this data that a signal from Toll somehow facilitates sequestration of pelle at the plasma membrane by tube where it can fulfil its signalling role. This could involve a signal dependent interaction of tube and Toll but yeast two-hybrid experiments show no signal independent interaction of the two.

Cactus is homologous to a mammalian gene IκB which encodes the inhibitory component of NF-kB (Kidd, 1992). Like IκB, cactus protein is cytoplasmic where it is bound in a complex with dorsal homodimers (Isoda and Nusslien-Volhard, 1994). Loss of cactus from this complex correlates with nuclear localisation Like IκB, cactus is rapidly degraded in response to signalling. Signal dependent degradation occurs by some direct mechanism as it does not require the presence of dorsal (Belvin *et al.*, 1995). Both dorsal and cactus are phosphorylated *in vivo*. However, in contrast with the mechanism of activation of NF-κB it is dorsal, rather than cactus phosphorylation which correlates with nuclear localisation (Whalen and Steward, 1994). Intriguingly, even null alleles of cactus do not completely abolish differential nuclear localisation of dorsal (Chasan and Anderson, 1993), indicating the existence of some cactus-independent mechanism of control, e.g.:- dependence of dorsal nuclear import on phosphorylation state.

1.2.2. Pattern specification by *dorsal*

Dorsal protein binds with differing affinities to a range of different promoter sites, with the consensus GGG(A/T)$_5$CCC. When bound it can act as either a repressor or activator of transcription depending on the site and its context (Jiang and Levine, 1993). This allows the gradient of dorsal nuclear localisation to set up domains of expression of various target genes across the D-V axis, which then go on to define which regions become (from ventral to dorsal) mesoderm, neuroectoderm,

FIG. 10:- Diagram illustrating model of Toll activation leading to activation of dorsal.



dorsal epidermis or amnioserosa. Perhaps the simplest example of the importance of site affinity in pattern specification by the dorsal protein gradient is seen in the control of *twist* expression. *Twist* encodes a helix-loop-helix (HLH) type transcription factor which is expressed in a steep *dorsal*-dependent gradient from ventral (high) to lateral (low). The pattern of expression of *twist* is due to a small region of the *twist* promoter known as a ventral activator region (VAR). This region was defined by its ability to drive early ventral transcription of a heterologous promoter (HSP-70) (Pan *et al.*, 1991). The VAR contains a number of essential low affinity dorsal binding sites. Variation of the number and affinity of *dorsal* sites in the VAR changes the pattern and the gradient of expression seen in reporter assays.

It should be noted, however, that there is no simple mapping between the domains of expression or repression of dorsal target genes and the affinity of the dorsal binding sites thought to be responsible for controlling expression. For instance, *zerknüllt* (*zen*) and *decapentaplegic* (*dpp*), which are both repressed ventrally by dorsal, have nearly identical domains of expression, but whereas repression of *zen* appears to be due to a small number of high affinity dorsal binding sites, repression of *dpp* is thought to be due to a larger number of weak *dorsal* binding sites (Huang *et al.*, 1993). What does seem to be important in controlling the

domain of expression of *dorsal* target genes is the context of the sites driving transcription. The sites necessary for ventral repression of *zen*, *dpp*, and *tolloid* (*tll*) are all present in ventral repression elements (VRE's). These are negative enhancers (or silencers), definable by their ability to overcome the activity of heterologous promoters independent of orientation and context, and by their ability to act over large distances (Huang *et al.*, 1993; Jiang *et al.*, 1993a; Kirov *et al.*, 1994). Cross species comparison of the *zen* VRE has identified conserved T-rich regions adjacent to the dorsal binding sites which have been shown by mutagenesis to be essential for VRE action, although the nature of the factors which bind these sites is still unknown. In fact mutagenesis of these T-rich sequences can be used to convert the *zen* VRE into an enhancer, illustrating that the choice of repression or activation is context and not site sequence dependent (Kirov *et al.*, 1993; Jiang *et al.*, 1993a). The existence of a similar repressor element adjacent to a κB site in the IFN-γ enhancer suggests that this repressor may be conserved in mammals (Nourbakhsh *et al.*, 1993). A maternal factor which can bind this T-rich sequence and is capable of converting dorsal into a repressor when bound to the *zen* VRE, as well as NF-κB bound to the IFN-γ enhancer, has recently been isolated in a functional screen in yeast (Lehming *et al.*, 1994). This factor, dorsal switch protein 1 (DSp1), has homology to a mammalian gene HMG I(Y) and may be involved in the potentiation of transactivation by NF-κB from the IL-2R promoter (John *et al.*, 1995).

Ultimately sharp domains of expression of dorsal target genes are specified by the interaction of dorsal with both transcriptional activators and repressors, some of which are dorsal targets genes themselves. One good example of this is the control of expression of the repressor protein *snail* in the mesoderm. Expression of *snail* in the mesoderm is required for the restriction of neurectodermal genes, such as *rhomboid* (*rho*), to the lateral regions, so a sharp lateral boundary of *snail* expression is required for precise definition of the mesoderm/neuroectoderm boundary. *Snail* is initially expressed weakly in only the ventral-most nuclei. The domain of *snail* expression then gradually moves laterally during the next cleavage cycle (nuclear division, not cellular cleavage), but still with a poorly defined lateral boundary. This is then quickly refined to give strong uniform expression throughout the mesoderm with a sharp lateral boundary. In *twi*$^{-}$/*twi*$^{-}$ mutant embryos this mature pattern fails to form (Kosman *et al.*, 1991). Analysis of the *snail* promoter has shown it to contain both dorsal and twist binding sites and it has been suggested that it is the sum of both the *dorsal* and *twist* gradients acting on this promoter that produces the strong and sharply defined late expression pattern of *snail* (Ip *et al.*, 1992a).

Expression of *dorsal* target genes in the lateral regions of the embryo (prospective neuroectoderm) where the nuclear concentration of *dorsal* protein is low requires both *dorsal* and HLH proteins. A good example of this is the control of expression of *rhomboid (rho). rho* expression is driven by an enhancer called the neuroectoderm enhancer (NEE), which contains a consensus *snail* binding site essential for ventral repression (Ip *et al.*, 1992b), four dorsal binding sites and at least five consensus E-boxes (bHLH binding sites). In reporter studies, the loss of four of these E-boxes abolishes all expression apart from a low level of ventral expression in the syncytial blastoderm. In a sna^{-}/sna^{-} background expression is restored ventrally but not laterally, i.e.:- the E-boxes are required for lateral expression (Ip *et al.*, 1992b). Exactly which HLH proteins are required is not currently known, however genetic studies show that embryos doubly or triply heterozygous for both *dorsal* and a number of bHLH protein encoding genes show severely abnormal neurectoderm development (Gonzalez-Crespo *et al.*, 1993). Also, dorsal protein has recently been shown to physically interact with various HLH proteins *in vitro*, including the products of the *twist* and *scute* genes, which also bind co-operatively with dorsal to DNA containing adjacent *dorsal* binding sites and E-boxes (Gonzalez-Crespo *et al.*, 1993). Intriguingly the interactions between rel family, HLH family members and *snail* related proteins may be conserved in mammals. Evidence for this comes from the fact that the κ immunoglobulin light chain enhancer (κ enhancer), which normally controls a temporal pattern of κ light chain expression during B-cell maturation, can be used to drive expression of a reporter gene in *Drosophila* to give an expression pattern almost identical to that of *rhomboid* (Gonzalez-Crespo and Levine, 1994). The κ enhancer does not show any overall homology with the *rhomboid* NEE, but like the NEE it does contain consensus snail binding sites and E-boxes adjacent to κB sites.

1.2.3. Summary

This account illustrates the potential of rel family members for specifying positional information in embryos. It is important to note, however, that D-V patterning of a *Drosophila* embryo at the syncytial blastoderm stage, is not in any obvious way an event directly comparable to the establishment and patterning of the D-V axis in *Xenopus*. In the former the germ layers are specified by the D-V patterning event itself, whereas in the latter patterning events are thought to take place in the prospective mesoderm (on receiving inductive signals from the vegetal region of the embryo). It could be argued that the situation in insects, where patterning of the

early axes takes place in a syncytial blastoderm, provides a situation where morphogen gradients can be easily produced and utilised for patterning. This is a rather different situation to that found in vertebrate embryos where most patterning events take place in a cleaved embryo where it is less easy to envisage morphogen gradients forming across large parts of the embryo by free diffusion of a ligand. However, as discussed later (see page 46), a series of elegant experiments carried out by John Gurdon's group do in fact provide evidence that a peptide ligand can form gradients in a *Xenopus* embryo by diffusion.

On the molecular level comparisons between the two systems are much stronger. A number of developmentally important signalling pathways found in *Drosophila* are now known to exist in vertebrates, e.g.:- hedgehog and wingless signalling pathways and, as discussed, the Toll - dorsal pathway does seem to be, in some ways comparable with the IL-1R - NF-kB pathway.

1.3. Models of mesoderm induction and patterning in *Xenopus*.

The aim of this section is to provide sufficient background for the discussion of phenotypes, and models of their origins, in later chapters. The section consists of an account of currently accepted models of pattern specification during the early stages of *Xenopus* development, and their experimental bases. The first part deals with classical models of this process, after a brief descriptive account of early development. The second part deals with models for the molecular nature of these processes. All stages referred to are as defined by Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

1.3.1. A brief descriptive account of early *Xenopus* development.

The unfertilised *Xenopus* egg is a single large cell with externally visible assymetry. One half of the egg, the animal hemisphere, is darkly pigmented due to a layer of pigment granules under the surface while the other half, the vegetal hemisphere, is a yellowish white, due to the high concentration of yolk granules it contains. The egg appears to be rotationally symmetrical about the animal-vegetal axis, and as discussed later (see page 31), this seems also to be true with respect to developmental potential. Internally the egg is divided into visually distinct cortical and inner cytoplasmic layers. After fertilisation the cortex rotates approximately 30° with respect to the inner cytoplasm, leading to the formation equatorially of a lightly pigmented arc on one side of the embryo, corresponding to pigmented animal cytoplasm underlying unpigmented vegetal cortex (in some amphibia, e.g.:- Rana, this is known as the grey crescent). As discussed later (see page 31), this arc marks the future dorsal side of the embryo. The direction of cortical rotation is determined by the site of sperm entry so that the arc of reduced pigmentation forms on the opposite side to the sperm entry point. Shortly following this event cleavage begins. During cleavage a cavity opens up in the animal hemisphere called the blastocoel (embryos from stage 7-9 are referred to as blastulae).

There is quite a high degree of topographic projection from the fertilised egg onwards. This combined with the fact that the first few cleavage planes usually follow a predictable pattern[6] has allowed the construction of probabilistic[7] fate[8] maps

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[6] It is important to note that no particular pattern of cleavage is **necessary** for normal development.

[7] The maps are probabilistic because of slight variations in the positioning of cleavage planes, and because of the extent of cell mixing during later stages.

by the labelling of individual blastomeres during early cleavage (e.g.:- Dale and Slack, 1987a). Briefly, such studies, along with later stage fate maps, have shown that the ectoderm arises from the animal hemisphere, with future epidermis arising mainly from the ventral half and future neural plate from the dorsal half. Mesoderm arises from a torus of material around the equator, but not from the surface layer, the dorsal part of which has an endodermal fate. The equatorial region can be further subdivided with mesodermal tissues arising from positions along the dorsal-ventral (D-V) axis roughly corresponding to their final D-V arrangement (following gastrulation), with head mesoderm also arising dorsally. The remaining endoderm arises from the vegetal hemisphere (summary from Slack, 1991).

During stage 9 the inner cells of the animal hemisphere undergo radial intercalation so that the animal hemisphere thins from 3-4 cells down to just two cell layers, leading to an expansion of the animal cap vegetally, and a convergence of animal cells in the marginal zone (the equator of the blastula). This process is known as epiboly. Gastrulation begins at stage 10 with the formation of a darkly pigmented arc called the dorsal blastopore lip, on the future dorsal side on the edge of the vegetal hemisphere. This lip then extends ventrally to form a complete circle (the blastopore). As gastrulation proceeds epiboly continues, leading to the closure of the blastopore, which ends up in a ventral, posterior position. During gastrulation the future mesoderm involutes and migrates over the blastocoel roof leading to the collapse of the blastocoel, and the formation of a second cavity, the archenteron, which will become the gut lumen. Involution is driven by a co-ordinated pattern of radial intercalation of prospective mesoderm (Shih and Keller, 1992a). Extension of the mesoderm occurs to the greatest extent on the dorsal side, with the leading edge reaching well past the animal pole, and is driven by convergence of dorso-laterally involuting mesodermal cells towards the dorsal midline by medio-lateral intercalation (Shih and Keller, 1992a). This process continues after gastrulation in the dorsal mesoderm and other tissues (Discussed in Keller, 1992) leading to lengthening of the embryo. The future head mesoderm behaves slightly differently. It is derived from the leading edge of the involuting mesoderm and becomes morphologically distinct early in gastrulation due to the fact that, instead of undergoing convergence and extension, it actively migrates over the blastocoel roof (Winklbauer and Nagel, 1991). The next stage of development is known as neurulation. During neurulation the neural plate (future central nervous system) forms from ectoderm overlying the

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[8] Note that **fate** refers to 'the future experience of a region of the embryo ..., [and] does not imply anything about the commitment of the region in question' (Slack 1991).

dorsal mesoderm, and rolls up to form the neural tube, overlain by epidermis arising from ectoderm adjacent to the neural plate.

1.3.2. Classical Models

Early development can be viewed as a process of symmetry breaking. The first such event in *Xenopus* development is cortical rotation which breaks the rotational symmetry around the animal-vegetal axis so that the resulting arc of reduced equatorial pigmentation attains a dorsal fate. The causal connection between the cortical rotation and future establishment of fate has been confirmed by experiments using inhibitors of microtubule polymerisation, such as U.V. irradiation shortly after fertilisation , to inhibit cortical rotation (Malacinski *et al.*, 1975). At their most extreme these treatments lead to the formation of rotationally symmetrical 'ventralised' embryos. The mechanism of action of the U.V. treatment used in these experiments is confirmed by phenotypic rescue experiments where treated embryos are immersed in a solution of Ficoll (a polysaccharide which osmotically removes water from under the vitelline membrane preventing free rotation), and then tipped, allowing the cortical rotation to occur under the influence of gravity (Scharf and Gehart, 1980).

In order to understand how cortical rotation might lead to the establishment of dorsal-ventral patterning it is important to understand the context into which such a patterning event must fit. One of the most important, and well accepted concepts of amphibian development is that, in order for mesoderm to form, inducing signals must pass from vegetal to equatorial cells. This phenomenon was initially discovered in urodeles with experiments which were later repeated by the same groups using *Xenopus*. Specification experiments performed by Nakamura and Takasaki using *Triturus* showed that explants from the equatorial region of the mid-blastula formed mesoderm in neutral culture, whereas explants from the same regions of 32-64 cell embryos became ciliated ectodermal cells (Nakamura and Takasaki, 1970). Taken together with work by Nieuwkoop and colleagues showing that blastula animal cap explants, which form 'atypical epidermis' when cultured alone, could be induced to form mesoderm when combined with vegetal explants (Nieuwkoop, 1969a), this lead to the hypothesis that mesoderm inducing signals from the vegetal hemisphere were essential for the induction of mesoderm equatorially. The original experiments demonstrating the existence of these signals involved combining blastula 'animal cap' explants with vegetal pole tissue of the same stage and scoring the mesoderm induced. In fact, heterochronic combinations of animal and vegetal explants have shown that

mesoderm induction is initiated during cleavage (Jones and Woodland, 1987). These experiments put the beginning of animal cap competence between stage 6 and 6.5 (64 cell stage) ending at around stage 10.5[9]. Secretion of the inductive signal may begin even earlier and is over by stage 11. The fact that mesoderm induction is already occurring in cleavage stage embryos means that, at least at these stages, mesoderm induction occurs independently of transcription, as zygotic transcription does not begin until the Mid-Blastula Transition (MBT) at about stage 8.5 (Newport and Kirschner, 1982).

Early models of the nature of the asymmetry established by cortical rotation were based on the results of investigations into the nature of the mesoderm induced in animal caps when combined with different regions of the vegetal hemisphere. Such experiments show that dorsal-ventral polarity is present in the vegetal hemisphere and is communicated to the prospective mesoderm by induction (Nieuwkoop, 1969(b)). This conclusion is backed up by the results of blastomere ablation and transplantation experiments. At the eight cell stage ablation of either of the two dorso-vegetal cells produces ventralised embryos (Kageura and Yanama, 1984). At the 32-cell stage dorsal blastomeres from both the vegetal-most tier and those from the overlying tier can rescue U.V. ventralised embryos when transplanted into their corresponding positions (Gimlich, 1986). As the vegetal-most dorsal blastomeres are fated not to become mesoderm, but to contribute to the endoderm, it has been suggested that these cells contain at least part of a vegetal dorsal signalling centre. Interestingly, at the eight cell stage substitution of either of the two ventro-vegetal blastomeres for a dorso-vegetal one leads to the formation of double axis embryos (Kageura and Yanama, 1986). The converse experiment, however, produces no phenotype. This result has been taken to imply that the signal emitted by the dorsal-vegetal signalling centre is dominant over the ventral inducing signal. This signalling region is now generally referred to as the Nieuwkoop centre.

This, however, is not the complete extent of dorsal/ventral patterning in the blastula. It has been known for some time that notochord formation can be induced by vegetal hemispheres in conjugates containing isolated dorsal but not ventral animal cap halves. This bias is also seen with respect to induction of animal cap halves by mesoderm inducers activin (when used externally) and bFGF (when injected as mRNA (see p.39)); (Sokol and Melton, 1991; Kimelman and Maas, 1992). In both

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[9]Note - Studies of induction with activin put the end of competence as later (S11.5), this may be because the Jones and Woodland study scored only dorsal and intermediate mesoderm.

cases the differential response of dorsal and ventral animal cap halves is not seen in animal caps from U.V. irradiated embryos. This demonstrates a causal connection between the 'competence pre-pattern' exhibited by the animal hemisphere and the cortical rotation. Interestingly, dissagregation of animal caps from U.V. ventralised embryos can restore competence to respond to high activin concentrations by producing dorsal mesoderm as long as the caps are re-aggregated before control stage 10 (Green *et al.*, 1994; also see page 45). Thus the competence prepattern appears to arise from the presence of a diffusible ventralising agent on the ventral side. The developmental significance of this pre-pattern currently remains obscure.

The molecular nature of the Nieuwkoop centre signal is hinted at by the teratogenic effects of lithium on cleavage stage embryos. Such treatment produces, as a most extreme limit form, rotationally symmetrical antero-dorsalised embryos. One of the major biochemical effects of lithium is to inhibit the enzyme inositol-1-phosphatase, which is essential for the re-synthesis of the pool of target lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) in the phosphoinositide signalling pathway. As the pool of PIP_2 in the plasma membrane available for signalling is limited this leads to an inhibition of the phosphoinositide signalling pathway (Berridge, 1989). That the teratogenic effects of lithium are due to this biochemical effect is confirmed by the fact that lithium has been shown to inhibit an increase in endogenous levels of the second messenger inositol-1,4,5-trisphosphate ($InsP_3$) occurring at the 32-64 cell stage (Maslanski *et al.*, 1992) and that the lithium phenotype can be rescued by injection of inositol (Busa and Gimlich, 1989).

The simplest interpretation of this data is that the lithium phenotype results from the inhibition of a signalling pathway, suggesting that an active ventral signal is required for induction of ventral mesoderm, and that dorsal mesoderm is formed as a default in its absence. That the limit form of the phenotype is not just dorsalised, but dorso-anteriorised could be taken to imply that the instructive nature of the Nieuwkoop centre is more complex than merely specifying dorsal mesoderm. This need not be the case as the fate maps indicate that while head mesoderm arises solely from the dorsal-most mesoderm of the blastula, the dorsal trunk mesoderm has a large contribution from laterally involuting cells due to medio-lateral intercalation. Presumably these cells achieve a more anterior fate in embryos where the entire marginal zone has been re-specified as organiser by lithium treatment. It is difficult to understand, however, how the same argument could be applied to explain the range of phenotypes, from headless to radially ventralised, resulting from U.V. treatment. Another implication of the phenotype is that, as the limit form is

rotationally symmetric, the competence pre-pattern of the animal cap appears to be either unnecessary for dorsal tissue formation, or dependent (directly or indirectly) on the signal inhibited by lithium.

Of course, talking simply in terms of dorsal and ventral fates is an oversimplification. Some patterning mechanism must be responsible for specifying intermediate fates. Interestingly, at the blastula stage the fate map differs significantly from the specification map[10] with respect to intermediate tissues. For example, 60% of somite is fated to come from the ventral marginal zone (Dale and Slack, 1987a), but when blastulae are divided in half, the ventral halves form only ventral mesoderm, and little or no muscle (Dale and Slack, 1987b). These experiments imply the presence of a 'dorsalising' signal emanating from the dorsal half of the embryo. Experiments using explant combinations have shown that this signal comes from tissue arising from an arc of approximately 60° in the dorsal most region of the marginal zone, and that dorsalisation itself takes place mainly during gastrulation. This idea fits well with one of the most famous experiments in amphibian embryology - the organiser graft (Spemann and Mangold, 1924). This experiment, originally done with newt embryos but easily repeatable in *Xenopus* (Smith and Slack, 1983), involves grafting the dorsal blastopore lip of a stage 10 embryo onto the ventral marginal zone of a donor embryo. This results in the formation of a double embryo, with the grafted lip contributing mainly to the notochord and prechordal plate of a secondary axis. Most importantly however, the graft 'organises' (dorsalises) the surrounding prospective ventral mesoderm to form the rest of the mesoderm of the secondary axis (i.e.:- intermediate mesoderm such as somites and lateral plate etc.). As the dorsal blastopore lip region and the earlier dorsalising centre of the blastula dorsal marginal zone are contiguous structures it seems sensible to consider them as different developmental stages of the same phenomenon (the organiser).

The events of mesoderm induction and dorsalisation are summarised by the three signal model (see figure 11) proposed by Slack and colleagues (Smith and Slack, 1983). In this model the mesoderm inducing signals emitted by the vegetal hemisphere consist of one found both dorsally and ventrally (signal 1), and a dominant second signal activated by cortical rotation (signal 2, also called the Nieuwkoop centre signal) which is present only dorsally. This asymmetry then

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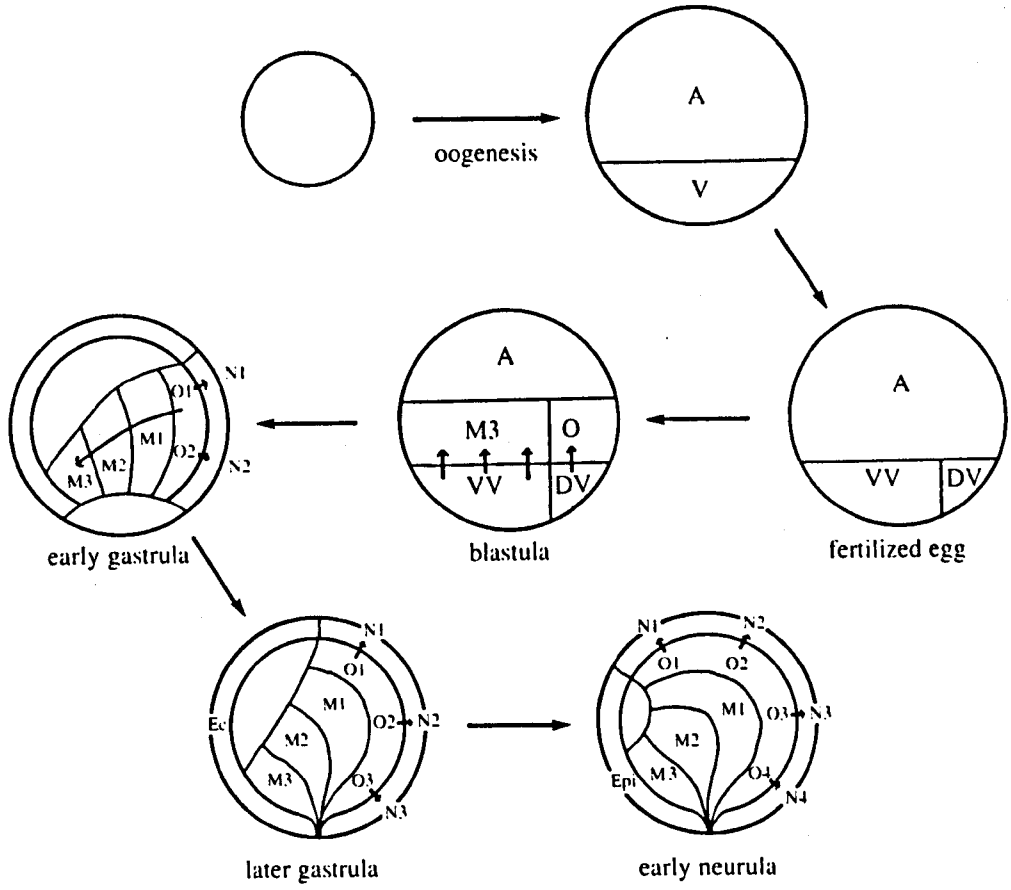
[10]Specification here is used to mean 'commitment of a commitment of a **tissue region** which is manifested on culture in a neutral medium but may still be reversible' (Slack, 1991)

induces the formation of a 'dorsalising centre' in the dorsal marginal zone which secretes the third signal. One way of reconciling this model with the implications of the lithium phenotype is simply to propose that one of the effects of signal 1 is to stimulate IP_3 levels, and that the sole purpose of signal 2 is as an antagonist of phosphoinositide signalling. This satisfies the criterion that signal 2 should be dominant, with lithium acting to mimic its effect. The three signal model, while providing a framework for future work, is probably an oversimplification. It is particularly important to bear in mind that each of the three signals could actually be a combination of different inducers and/or different concentrations of the same inducing agent, and that the same inducing agents may have different developmental effects at different stages of development. It is also important to bear in mind that the actual time windows for particular inductive events may be much tighter than those suggested by classical experiments with limited resolution.

Kimelman and colleagues, synthesising data from a variety of sources, have proposed a more detailed model of organiser action than that outlined in the three signal model (Kimelman *et al.*, 1992). A comparison of the fate map at 32 cells with the specification map at 128 cells shows a significant difference with respect to the region which will contribute to notochord. The fate map shows animal cells from close to the marginal zone forming notochord, but the specification map shows that this state of specification has not been achieved by the 128 cell stage (Dale and Slack, 1987(a) and (b)). In fact, notochord specification seems to be due to an inductive signal secreted by the lower DMZ. The evidence for this comes from some elegant experiments combining normal and U.V. ventralised stage 9 embryos cut between the upper and lower DMZ which show that lower DMZ from a normal embryo can induce ventralised upper DMZ to form notochord (Stewart and Gerhart, 1991). The lack of secondary notochord induction in Spemann organiser grafting experiments shows that the ability of DMZ to induce notochord formation (or the competence to respond to those signals) has disappeared by stage 10 even though the ability to dorsalise ventral mesoderm (or the competence to respond) is obviously not lost. Collectively this has lead Kimelman and colleagues to propose that the role of the blastula organiser (in the lower DMZ) is to induce notochord (in the upper DMZ), and that the prospective notochord then acts as a source of the late organiser signal, dorsalising the ventral and lateral mesoderm as it converges on the dorsal mid-line during gastrulation (see page 30). The lower DMZ becomes the prospective head mesoderm (head organiser ?) during gastrulation.

FIG. 11:- The Three Signal Model.



The three areas A (animal), VV (ventro-vegetal) and DV (dorso-vegetal) are set up in the egg as a result of oogenesis and the postfertilisation cortical rotation movements. In the blastula stages, the DV region emits a signal which induces the organiser (O) and the VV region emits a signal which induces default, or ventral type, mesoderm (M3). During gastrulation, the organiser emits a third, graded, signal, termed dorsalisation, which regionalises the mesoderm into zones forming somite (M1), lateral plate (M2) and blood islands (M3). Finally, neural induction by the archenteron roof is shown. This occurs progressively during gastrulation (Figure from Slack, 1991).

Organiser grafted embryos frequently form, not just a second trunk, but a complete double embryo including a head. This obviously suggests some element of antero-posterior pre-patterning exists (implicitly or explicitly) in the dorsal blastopore lip (DBL) at stage 10. The extent to which such patterning can occur is limited by the extent of cell mixing which occurs during gastrulation. As the DBL in *Xenopus* is small and the prospective mesoderm lies below the surface, detailed fate mapping is technically very difficult. Equivalent experiments carried out in the urodele *Cynops* show a projection of approximately head-trunk-tail resolution (Okada and Hama, 1945). However, when the state of specification of these regions was investigated by

culturing the appropriate regions wrapped in ectoderm, the regions fated to become head and trunk both formed dorsal axial mesoderm, and the prospective tail formed only epidermis (Hama *et al.*, 1985). Thus, the 'pre-patterning' of this region is implicit, presumably requiring the correct tissue movements in its normal embryological context to form the right structures. One example of how this may occur, comes from work by Ray Keller on the organising properties of the epithelium of the DBL in *Xenopus*. As already mentioned, in *Xenopus* the surface 'epithelial' layer of the dorsal marginal zone (DMZ) has an endodermal fate, and does not mix with the deep prospective mesoderm. However, organiser grafts can be successfully carried out using this tissue, leading to the formation of a secondary trunk, but no head structures (Shih and Keller, 1992b). In fact the direction of convergent extension of mesoderm induced by epithelial DMZ grafts is dependent on the orientation of the graft. This suggests a role for this layer in organising directed convergent extension, Interestingly, during normal development, the epithelial layer loses contact with the prospective head mesoderm (which does not undergo convergent extension) early in gastrulation (stage 10.5). The timing of this event correlates quite precisely with the onset of differential head/trunk marker expression (see page 38) and soon afterwards (stage 11) head mesoderm becomes morphologically distinct from prospective axial mesoderm (see page 30).

Anterior-posterior (A-P) patterning in the other two germ layers is thought to arise from signalling from the mesoderm during and after gastrulation, although comparatively little is known about this process in endoderm (reviewed in Slack, 1991). The ability of mesoderm to induce and pattern neural tissue from the animal hemisphere (reviewed in more detail in Slack and Tannahill, 1992) was first indicated by the fact that secondary axes arising from organiser grafts have a secondary nervous system with correct A-P patterning[11]. This patterning occurs by a combination of at least two types of signal. Explant combinations have been used to show that mesoderm can induce the formation of neural tissue with positional specification slightly anterior to its own (reviewed in Slack and Tannahill, 1992). This is known as vertical or appositional induction. It is also possible that a series of vertical signals received during gastrulation as the dorsal mesoderm moves underneath the prospective neural plate are important for patterning (Sivè *et al.*, 1989) It has also become clear recently that some neural induction and patterning can occur via inducing signals travelling through the plane of the ectoderm from the organiser during gastrulation (Doniach *et al.*, 1992; Ruiz-i-Altaba, 1992). This process is known as planar

[11] This arises from ventral animal cap whose normal fate is epidermal.

induction. It seems likely that the final A-P positional character of the neural plate is a result of a combination of vertical and planar inductive signals, possibly along with further signalling within the neural plate itself.

It is interesting to note that the expression patterns one might predict for neural inducers and organiser signals are essentially similar

1.3.3. The molecular nature of mesoderm induction and patterning

For the sake of simplicity in this section I shall deal almost exclusively with the molecular nature and activity of candidates for endogenous inducing factors, leaving discussion of transduction of such signals, and specification activities of transcription factors to the relevant sections of the results and discussion chapters. Non secreted gene products will only be discussed as markers of specification. Encouragingly, the expression patterns of almost all of the mesodermal markers expressed in the late blastula cloned so far conform to what might be predicted by the three signal model. Markers can be divided into three groups based on their blastula expression patterns. They are either pan-mesodermal, such as *Xbra* and *snail*, organiser specific such as *Goosecoid* (*gsc*), *Xlim*, *pintallavis* and *noggin*, or ventrally expressed (everything but the organiser) such as *Xwnt-8* and *Xpo*. *Xnot* expression is initially ubiquitous (stage 9), but gradually becomes restricted, first to the mesoderm and then to the organiser region. By stage 10.5 it is localised within the organiser to non-involuted cells not adjacent to the lip (Von Dassow et al, 1993). It appears to be a very early marker of prospective trunk, but not prospective head regions of the organiser, and as such suggests that specification of head and trunk mesoderm has begun by stage 10.5.

Inducing agents

There are currently three experimentally testable requirements which must be fulfilled by a factor in order to qualify as a potential mesoderm inducer. The first of these is mesoderm inducing activity as tested in an animal cap assay. Such experiments can take the form either of direct treatment of animal cap explants with soluble protein, or of auto-induction of caps excised from embryos injected with a specific transcript. These experiments also provide evidence of the type of inducing activity possessed by the factor, although competence pre-patterning of the cap

complicates the interpretation of such data. In addition to experiments of this form, overexpression experiments in whole embryos can frequently give clues to the potential *in vivo* role(s) of an inducing agent. The second requirement is that the potential mesoderm inducer be expressed, in an active form, in the right place at the right time as suggested by the classical models, e.g.:- the three signal model. It should be noted however that expression patterns do occasionally suggest activities not previously predicted. Finally, the third experimental requirement, which provides evidence that a factor may be necessary rather than merely sufficient for an inductive event, is that inhibition of signalling by the factor in whole embryos produces a phenotype consistent with the factor's predicted role. Such evidence comes from the expression of dominant inhibitory mutants of receptors or ligands[12]. The problem with this form of experiment is that the specificity of oligomerisation of receptors used in these experiments, with related receptors or other signalling components, is not currently well understood.

All mesoderm inducers currently characterised are members of the FGF or TGFβ super-families of peptide growth factors (PGF's) and can be grouped into four classes with distinct activities. Incubation of animal cap explants with members of the FGF family only induces formation of ventral type mesoderm in animal caps (Slack, 1987). However, in auto-induction assays with bFGF animal caps form both dorsal and ventral mesoderm, presumably because of longer and/or earlier exposure of the induced cells (Kimelman and Maas, 1992). TGFβ2 and activin, both members of the TGF-β superfamily, can induce a range of mesodermal types from ventral at low concentrations, through dorsal axial mesoderm at intermediate concentrations to head mesoderm at the highest concentrations (Green and Smith, 1990; Green et al., 1990). The complex nature of this dose response and its possible significance will be discussed later. The cloning of a maternally expressed *Xenopus* member of the TGFβ superfamily named Vg-1 around the same time as the inducing activity of activin was discovered raised hopes that an endogenous mesoderm inducer had been found (Weeks and Melton, 1987). Initially, however, no inducing activity, by injected mRNA or by purified protein, could be detected. Further investigation showed that the exogenous protein was not being processed *in vivo* into a (predicted) active form[13]. This problem was eventually overcome by two separate groups using the same

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[12]For a discussion of the design and action of dominant negative PGF-R's see chapter 6

[13]All TGFβ family members are thought to require dimeristaion and proteolytic cleavage to generate the active form.

approach of fusing the predicted active region of Vg-1 to the pre-proregion of a more readily cleaved member of the TGFβ superfamily. The results obtained by these two groups appear, however, to be conflicting. A Vg-1/BMP-2 fusion generated by Thomsen and Melton containing the BMP-2 proteolytic cleavage site can induce the whole range of mesodermal tissues in auto-induction assays (Thomsen and Melton, 1993). Another fusion, made by Dale and colleagues, using the cleaved region of BMP-4, but this time retaining the Vg-1 cleavage site, has only ventral and lateral mesoderm-inducing activity (Dale *et al.*, 1993). Whether this difference simply reflects a difference in the maximum amount of cleaved product that can be generated, or some more fundamental difference such as dimerisation specificity is not yet clear. It is important to note that the activity of the Vg-1/BMP-2 fusion in axial rescue assays (see below) clearly makes processed Vg-1 activity distinct from that of activin. Finally, BMP4 and BMP2, also members of the TGFβ superfamily can induce extreme ventral mesoderm in auto-induction assays (Dale *et al.*, 1992), and as protein secreted by transfected COS cells (Koster, 1990). Somewhat suprisingly for a putative ventral mesoderm inducer, it has been shown that BMP-2/-4 induction of ventral mesoderm is dominant over activin (Jones *et al.*, 1992).

Animal cap assays only give an idea of the type of mesoderm which can be induced in isolation by a particular factor. An idea of how these factors might act *in vivo* can be gleaned from the results of whole embryo expression studies For example, factors which can completely rescue axial development in U.V. ventralised embryos when 'unilaterally' injected, or can induce a complete second axis by ventral injection, are candidates for the Nieuwkoop centre signal, and/or for a complete organiser signal. The only characterised mesoderm inducer which falls into this category is Vg-1 (in the form of Thomsen and Melton's Vg-1/BMP-2 fusion). In the same assay activin only rescues trunk development (Steinbeisser *et al.*, 1993), suggesting, if the three signal model holds, that it could be a component of the organiser signal, but is not a Nieuwkoop centre signal candidate. Injection of mRNA encoding BMP-2 or -4 seems to have the opposite effect to activin in that dorsal injection of normal embryos leads to ventralisation (Dale *et al.*, 1992; Jones *et al.*, 1992). Interestingly embryos ventralised in this fashion differ from embryos ventralised by U.V. treatment in that they form what looks like a normal blastopore. This has been taken to imply that BMP-4 acts late to ventralise mesoderm, possibly during gastrulation (Dale *et al.*, 1992). A dominant ventral mesoderm inducing activity such as this is seems to conflict directly with the classical data. It is difficult to understand how organsiser grafts can induce a second axis in the presence of a late

acting dominant ventralising signal. A possible resolution of this apparent paradox is discussed later (see page 48).

Mapping the distribution of active PGF's is not a simple matter. Although the distribution of transcripts can be assayed easily by in situ hybridisation or RNase protection assay, mapping the distribution of secreted PGF's has proved more difficult. The situation is complicated in the case of TGF-β family members by their requirement for post-secretion processing (involving a specific protease) in order to be active. Unfortunately no data so far exists on the distribution of active forms of TGF-β family members, or of proteases capable of cleaving them. An additional complication with FGF family members is that the effective affinity of ligand for receptor can be positively or negatively regulated by the local concentration of different proteoglycans (Mason, 1994).

A number of forms of FGF are found in the early *Xenopus* embryo. aFGF (FGF-1) and bFGF (FGF-2) were early candidates for endogenous mesoderm inducers, as they are both expressed maternally (Slack and Issacs, 1989). The distribution of secreted bFGF and aFGF can not be easily mapped, however intracellular aFGF and bFGF are not localised. As neither of these molecules contains a signal sequence their secretion *in vivo* was initially thought to be doubtful. More recently, evidence has accumulated that the lack of a signal sequence need not prevent secretion under all circumstances (reviewed in Mason, 1994). Consequently, some form of controlled secretion of bFGF and/or aFGF cannot be ruled out. The fact that injected bFGF mRNA can induce mesoderm formation in animal cap explants (Kimelman and Maas, 1992) shows that secretion is at least possible, but this effect is only seen at highly non-physiological expression levels (Thompsen and Slack, 1992).

Another hopeful candidate for an endogenous FGF family mesoderm inducer, complete with signal sequence, is *Xenopus* embryonic FGF (XeFGF), which is most closely related to mammalian FGF-4 and FGF-6 (Issacs *et al.*, 1992). XeFGF mRNA is expressed at low levels maternally and at much higher levels zygotically, peaking during gastrulation. *In situ* hybridisations have shown that the zygotic expression is initially localised to the dorsal blastopore lip. This expression then extends completely around the forming blastopore, before becoming concentrated dorsally in both mesoderm and ectoderm in the posterior of the forming axis during the gastrula and neurula stages. At the tailbud stage, expression becomes limited to the tailbud. XeFGF has been shown to be part of an autoregulatory loop with the mesodermal

marker *Xbra*[14], so that overexpression of either in animal caps will induce expression of the other (Isaacs *et al.*, 1994). This suggests a role for XeFGF in maintenance of mesodermal fate during gastrulation. This is backed up by marginal zone dissagregation experiments showing that XeFGF can substitute for an endogenous factor lost in dissagregated culture which is necessary for the maintenance of *Xbra* expression in these cells during gastrulation, and that it has this activity after the ability of FGF to induce mesoderm formation in animal caps is lost.

The strongest evidence for an *in vivo* role for FGF in mesoderm induction comes from the phenotypic effects of overexpression of a dominant negative FGF receptor known as XFD. FGF receptors belong to the tyrosine kinase family of transmembrane receptors, and like other members of that family undergo ligand dependent dimerisation leading to auto-phosphorylation (reviewed in Mason, 1994). Phosphorylation occurs on multiple tyrosine residues, turning them into binding sites for SH2 domain containing proteins such as Phospholipase C-γ (PLC-γ) and the ras recruiting factor Grb-2, which are then phosphorylated by the receptor. XFD is a truncated form of the *Xenopus* FGFR-1 (also called flg-1), which lacks the intracellular tryosine kinase domain. It can completely abolish the response of *Xenopus* oocytes expressing exogenous wild type receptor to FGF treatment, as assayed by the ability of FGF to cause an increase the intracellular calcium concentration (Amaya *et al.*, 1991).

The penetrance of the XFD phenotype is variable (possibly due to limited diffusion of RNA targeted for translation on the rough endoplasmic reticulum (rER)). The most extreme embryos have extreme trunk deficiencies, showing little or no development of somites or notochord but develop completely normal heads (Amaya *et al.*, 1991). Ventrally, they usually form hearts, but these are generally abnormal, and blood is found ectopically or not at all. Gastrulation in these embryos is abnormal, with incomplete lateral and ventral invagination and involution, resulting in failure of the blastopore to close and its positioning dorsally. It is often difficult with such phenotypes to separate cause from effect when trying to dissect effects on mesodermal specification from effects on gastrulation. However, analysis of the mildly affected embryos has shown that, even when gastrulation proceeds apparently normally, muscle and notochord formation is impaired (Amaya *et al.*, 1993). Moreover, this effect appears to be direct, as lineage marking experiments involving co-injection of *lacZ* mRNA show a precise correlation between muscle defects and

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[14]See chapter 7 for discussion of Xbra properties & expression.

XFD expression. Analysis of early marker expression has shown that, consistent with a role for XeFGF in controlling its expression, *Xbra* expression is inhibited by XFD. The fact that expression of the immediate early lateral and ventral mesodermal marker *Xpo* is also inhibited also suggests an essential role for some form of FGF in the specification of ventral mesodermal fate. Not suprisingly maybe, expression of *goosecoid*, an early dorsal lip marker which becomes a marker of prospective head mesoderm during gastrulation, is unaffected.

The situation with regard to the precise nature of endogenous activin like signalling molecules is more ambiguous. Activins are active as cleaved homo or heterodimers of activin βA and βB chains ($(\beta A)_2$ is referred to as activin A, $(\beta B)_2$ as activin B and (βA/βB) as activin AB). Zygotic expression of activin βB begins in the blastula, whereas activin βA expression commences towards the end of gastrulation (stage13.) Although no activin transcripts are present maternally there is evidence for the presence of a maternal activin protein from activity purification experiments (Asashima *et al.*, 1991). The dimeric nature of activins makes them ideal targets for dominant negative interference clones. Although no experiments with such inhibitors have been carried out to date in *Xenopus*, an elegant set of activin dominant negative experiments have been carried using the teleost *Oryzias latipes* (Japanese Medaka), which distinguish between the contributions of maternal and zygotic activin (Wittbrodt and Rosa, 1994). Two dominant negative constructs were used. One of these, an uncleavable activin variant could inhibit mesoderm induction by co-expressed activin, presumably by forming an inactive dimer, but not by exogenous activin. Overexpression of this variant produced no phenotype. However, overexpression of the other activin dominant negative, which was thought to act as an antagonist of the activin receptor, produced non gastrulating embryos completely lacking mesoderm. The conclusion then is that, at least in the Medaka, neither maternal nor zygotic activin transcripts are necessary for mesoderm induction, but maternal activin protein, and/or something which acts through the same receptor(s) is.

Activins can also be specifically inhibited using follistatin, which is known to inhibit activins B and AB by binding to the activin β chain, and can inhibit some, but not all activities of activin A (e.g.:- Mather *et al.*, 1993). Overexpression of follistatin in embryos at levels which Schulte-Merker and colleagues calculate should be capable of inhibiting predicted endogenous levels of activin activity produces completely normal embryos (Schulte-Merker *et al.*, 1994). Therefore, they conclude, activins AB and B are not essential for mesoderm induction. However, it is interesting to note that at high levels (2ng down to 500pg) follistatin mRNA is 'toxic',

causing lethality at late gastrula, although gastrulation itself appears to proceed normally. Consistent with the theory that activin βB expression is unnecessary for mesoderm induction or patterning, mice in which the activin βB gene has been disrupted form mesoderm normally (Vassalli *et al.*, 1994).

Vg-1 is a much clearer candidate for a maternal endogenous mesoderm inducer. During oogenesis maternal Vg-1 transcripts become localised in a tight band close to the plasma membrane in the vegetal hemisphere, extending right up to the equator (Weeks and Melton, 1987; Kloc and Etkin, 1995). After fertilisation the RNA is released and diffuses to form a fuzzy vegetal band before being irreversibly localised by cleavage. This obviously puts Vg-1 in the right place at the right time to be either signal 1 of the three signal model, or if processing is dorsally localised, a component of the Nieuwkoop centre signal. However, it is important to note that processed Vg-1 has not been detected *in vivo* to date.

The results of experiments using Δ1XAR1, a dominant inhibitory form of the *Xenopus* activin type II receptor XAR1, are a good illustrations of the problems of the dominant negative approach. As with the dominant negative FGFR the penetrance of the phenotype is highly variable, however, the most extreme examples form no mesoderm whatsoever, and show no signs of gastrulation (Hemmati-Brivanlou and Melton, 1992). This was initially taken as evidence that activin, or at least something acting through the same receptor, is essential for all mesoderm induction. However, it is now known that Δ1XAR1 can inhbit induction of mesoderm in animal caps by bVg-1 and BMP-4, as well as by activin (Schulte-Merker *et al.*, 1994; Wilson and Hemmati-Brivanlou, 1995). As BMP-4 is thought to be late acting, the effect may be an indirect one on competence. This result is difficult to reconcile with the fact that the Δ1XAR1 phenotype can be rescued by the co-expression of wild-type XAR1, which suggests that the targets of Δ1XAR1 are at least functionally equivalent to XAR1. Interpretation of these results will obviously remain a problem until the interactions and activities of TGFβ receptors found in the early *Xenopus* embryos are better characterised. Because of the ambiguity surrounding the nature of the signal(s) blocked by Δ1XAR1 candidates will be referred to as 'activin-like' for the rest of this discussion.

One of the more striking implications of the limit form of the Δ1XAR1 phenotype is that the signal(s) which it blocks are necessary for the induction of mesoderm by FGF to occur *in vivo*. Mesoderm induction by FGF in animal caps is not inhibited by expression of Δ1XAR1 so this is not due to an effect on competence.

This would seem to leave effects on the control of translation or secretion (not to my knowledge investigated) which could take place pre-MBT, or more simply, control of expression, XeFGF being a candidate, which of course must be post MBT. In fact the relationship between the roles of the 'activin-like' signal and FGF in mesoderm induction is complicated. XFD can block the induction of a number of markers by activin in animal caps, suggesting an essential role for a secondary FGF signal (e.g.:- XeFGF) in this process (Labonne and Whitman, 1994; Cornell and Kimelman, 1994). Recent evidence suggests that the relationship between FGF and activin signals may be even more tangled. XFD can also block the induction of immediate early genes such as *Xbra* by activin (in the presence of cycloheximide), suggesting a requirement for a prior or concomitant FGF signal (Labonne et al, 1995). This may at first seem difficult to reconcile with the fact that the Δ1XAR1 phenotype suggests that activin is required for mesoderm induction by FGF. The resolution of this problem may lie in the observation that animal caps contain sub-inducing levels of activated MAPK which is inactive in animal caps expressing XFD. It therefore seems likely that the prior or concomitant FGF signal in caps is a sub-inducing, permissive signal, rather than the instructive inducing signal which is a component of the second signal.

From investigations of the complex dose response to activin it is clear that activin is a very versatile mesodermal patterning agent. The most fruitful method used to investigate the dose response to activin has been to use dissaggregated animal cap cells which are washed and re-aggregated following treatment. The resulting tissue can then be cultured until a control stage convenient for measuring the expression levels of relevant markers. All mesodermal markers assayed at control stage 10.5, after treatment of dispersed cells for an hour and re-aggregation prior to S10, show a broad dose response with expression quickly reaching a plateau as the activin concentration is raised. If however, the same markers are assayed at control stage 17 tight windows of expression are seen (Green *et al.*, 1994). For example, *Xwnt-8* is expressed only in response to low concentrations of activin, *Xbra* only in response to low to intermediate concentrations, and Gsc only in response to high concentrations. This profile corresponds well to the results of the original animal cap induction experiments with activin which showed that head structures were induced at the highest concentrations, dorsal axial ones at intermediate concentrations and ventral mesoderm at the lowest. The later, refined expression pattern could result either from intercellular interactions after stage 10.5 or by some cell autonomous mechanism. In fact, experiments in which the cells are kept in a dissaggregated state show that cell-cell interaction is essential even for the maintenance of expression of

most markers. FGF in some form is an obvious candidate for at least a component of this secondary signal.

An intriguing set of experiments involving re-aggregation of mixed populations of animal cap cells treated with different doses of activin suggests that secondary interactions following re-aggregation allow cells to 'poll' for their fate (Wilson and Melton, 1994). For example, mixtures of high dose treated cells with low dose treated cells form dorsal axial mesoderm expressing *Xbra*. If these cell populations had been kept separate the high dose treated cells would have expressed the head mesodermal marker Gsc and the cells receiving a low dose the ventral marker *Xwnt-8*. This could potentially solve the problem of how cells might accurately respond to an *in vivo* gradient of activin, but in itself, it does not constitute evidence for the existence of such a gradient.

A set of rather elegant experiments by John Gurdon and colleagues has provided evidence that activin is capable of forming a gradient by some form of (active?) diffusion from a source across non-expressing tissue. They carried out a series of experiments involving the combination of lineage labelled animal caps with vegetal explants from embryos over-expressing activin (Gurdon *et al.*, 1994). When the resulting conjugates were stained for expression of the pan-mesodermal marker *Xbra*, which is induced by medium but not high concentrations of activin, a band of expression was seen up to 6-cell diameters from the source tissue, with no expression seen in the intervening tissue. Gsc on the other hand, which is induced only by higher concentrations of activin, produces a band of expression including immediately adjacent cells, but which does not extend as far as the outer border of *Xbra* expression. These results are consistent with the formation of a gradient of activin by diffusion from the over-expressing tissue. The fact that this tissue can be successfully replaced by an activin soaked agarose bead shows that no extra signal is needed from the inducing tissue. It also seems that the result does not stem from the action of some 'relay mechanism' in which activin induces its own expression (or that of some other 'activin-like activity'), as the same result is obtained if the two explants in the conjugate are separated by a 'neutral' tissue (*Xenopus* gut) which has been pre-treated with cyclohexamide to inhibit translation.

Another way in which activin (or some activin-like molecule) could act as a patterning agent is suggested by the data on how animal cap competence changes with stage. Animal cap cells can only be induced to form notochord by high doses of activin prior to control stage 10. Between control stages 10 and 11 the same dose

induces animal caps to form muscle, and between control stages 11 and 11.5 only lateral and ventral mesoderm can be induced. After stage 11.5 competence is lost completely (Kimelman *et al.*, 1992). This change in competence is suggestively close to the actual sequence of inductive events attributed to the organiser at different stages of early development (see page 35). This has lead Kimelman and colleagues to propose that organiser action could be explained in terms of the secretion of activin, or an 'activin-like activity' at a constant level, with patterning occurring via the change in competence of the tissues coming into contact with the signal during development (Kimelman *et al.*, 1992).

BMP-4 seems to fulfil all the current experimental criteria for being an endogenous mesoderm inducer. It is expressed at low levels maternally and subsequently at much higher levels after MBT. As might be expected of a putative dominant inducer of ventral mesoderm zygotic expression is localised to the ventral marginal zone by S10 (Fainsod *et al.*, 1994). Injection of a dominant negative form of a *Xenopus* BMP-2/4 binding receptor into U.V. ventralised cleavage stage embryos rescues axis formation to a limited degree (Graff *et al.*, 1994). Significantly, the rescued axes do not contain notochord, consistent with BMP-4 being necessary for ventral mesoderm formation, but being active from stage 10 onwards, i.e.:- after notochord specification usually takes place. The recent report of the phenotype of BMP-4 null mice suggests an even broader role in mesoderm induction than may be the case for *Xenopus* (Winnier *et al.*, 1995).

One rather novel model has been proposed for BMP-2/4 action in the late blastula/early gastrula based on the observation that dominant negative BMP-2/4 receptor expressed in the animal cap alone (as judged by lineage marking by co-injection with *lacZ* mRNA) can dorsalise ventral marginal zone (Maeno *et al.*, 1994). This result, along with classical experiments showing that association of gastrula stage animal cap with prospective ventral mesoderm is required for proper ventral mesoderm differentiation (Maeno *et al.*, 1992; Tiedemann, 1993 (using *Ambystoma mexicanum*) suggests that endogenous BMP-4, expressed in the animal cap is required for normal ventral mesoderm differentiation.

The presence of BMP4 in the animal cap at sub-inducing levels may also have other effects. Graff and colleagues note that overexpression of a dominant negative BMP-2/4 receptor causes dorso-anteriorisation of the response of animal caps to activin so that, for example *Xwnt-8* expression is decreased and Gsc expression increased (Graff *et al.*, 1994). This could provide a solution to the problem of animal

cap 'competence pre-patterning', with sub-inducing levels of ventrally localised/active BMP-4 (which is known to be dominant over activin) being the diffusible ventral patterning agent whose presence was predicted by Green and colleagues (Green *et al.*, 1994; also see page 33). This could be simply tested by repeating the above experiment using dissected animal cap halves. This of course, still leaves the nature of the causal connection between cortical rotation and competence pre-pattern unsolved.

There still seems to be somewhat of a paradox here with respect to how organiser grafts or DMZ grafted to VMZ are able to dorsalise tissue expressing a dominant ventralising factor. Remember, BMP-4 can ventralise whole embryos when injected dorsally, implying dominance over the whole range of organiser signals. The answer may lie in the fact that overexpression experiments swamp any mechanisms which may regulate the injected factor at the transcriptional level. Consistent with this hypothesis BMP4 is expressed in all of the marginal zone except the organiser at stage 11-11.5, but by stage 13-14 has become restricted to the ends of the lateral plate and the ventral third of the slit blastopore (Fainsod *et al.*, 1994). However no evidence of transcriptional control of BMP-4 by dorsalising factors has so far been published.

BMP4 also has a second inducing activity, apparent at much lower concentrations. This is the ability to induce epidermis. Although animal cap explants form atypical epidermis as a default state when cultured in neutral medium, animal cap cells have a default neural state when subjected to prolonged dispersal in neutral medium (Slack, 1991). Animal cap explants taken from embryos expressing the dominant negative activin receptor Δ1XAR1 are also neuralised. This was initially taken as evidence for a role for activin in supressing neural fate. However as Δ1XAR1, directly or indirectly, also inhibits mesoderm induction by BMP-4 in animal caps, and animal caps express BMP-4 at sub-inducing levels, this would also seem a likely target. In confirmation of this hypothesis it has recently been confirmed that BMP-4 can induce epidermal fate (i.e.:- supress auto-neuralisation) in dissociated animal cap cells at concentrations 1000 fold lower than those required to induce mesoderm formation in whole animal caps (Wilson and Hemmati-Brivanlou, 1995)

Pattern modifying agents

As well as signalling molecules capable of directly inducing mesoderm a number molecules are known to be capable of modifying mesodermal patterning once

induction has occurred. One such modifying activity is exhibited by a number of members of the wnt family of signalling molecules. The family of genes to which these molecules belong includes the *Drosophila* gene *wingless* (*wg*), the mammalian proto-oncogene *Wnt-1*, and a whole array of recently cloned *Xenopus* members. Interest in the possible role of wnt family members in mesodermal patterning originates from experiments showing that ventral injection of mRNA encoding *Wnt-1* or *Xenopus wnt* family member *Xwnt-8* into marginal or vegetal blastomeres at the 8-16 cell stage produces embryos with a double axis (McMahon and Moon, 1989). *Xwnt-8* RNA injection can also be used to rescue embryos ventralised by exposure to UV prior to the first cleavage (Sokol *et al.*, 1991). As the injected cells do not necessarily contribute to the secondary or rescued axis, *Xwnt-8* appears to be causing injected cells to act as a Nieuwkoop centre, with *Xwnt-8* either acting directly to induce a Spemann organiser in adjacent cells or inducing the injected cells to secrete such a signal. This activity appears to be at odds with a gene normally expressed only zygotically, and even then in a pattern mutually exclusive with the organiser [15] (Smith and Harland, 1991). This apparent paradox could be resolved by supposing the existence of a maternal wnt or 'wnt like factor' present in the organiser or Nieuwkoop centre at early stages, which is mimicked by exogenous dorsal expression of *Xwnt-8*. A recently isolated maternally expressed member of the wnt family (Xwnt-11) seems to fit the criteria for being the endogenous wnt-like Nieuwkoop centre signal (Ku and Melton, 1993). Xwnt-11 transcripts are vegetally localised in the egg, but instead of the broad pattern of localisation seen with Vg-1, transcripts are sequestered in a small region at the vegetal pole prior to fertilisation (Kloc and Etkin, 1995). Currently nothing has been published on whether this distribution, or that of its protein product changes with cortical rotation. Endogenous *Xwnt-8* seems to have the opposite role to the Nieuwkoop centre signal after MBT when it appears to act as a ventralising signal. Evidence for this comes from experiments using an expression vector to mis-express Xwnt -8 in the organiser after MBT, which leads to the formation of ventralised embryos. *Xwnt-8* can also ventralise the response of animal caps to the dorsal mesoderm inducer activin when expressed after MBT (Christian and Moon, 1993).

Although the biochemical nature of wnt signalling is still unknown there are suggestive parallels between the actions of *Xwnt-8* and lithium. Both are dorsalising agents which can induce ectopic Nieuwkoop centre formation by ventral-vegetal injection during cleavage. However after MBT they both become ventralising agents. Both *Xwnt-8* and lithium can also act to dorsalise the response of animal caps to FGF

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[15] See section 7.1.2. for more detailed discussion of the expression pattern.

(Christian *et al.*, 1992; Slack *et al.*, 1988) and to low levels of activin (Sokol and Melton, 1992; Cooke *et al.*, 1989). As might be predicted these effects require preMBT treatment. These parallels have been used to suggest that *Xwnt-8* may act to suppress inositol phosphate metabolism (Slack, 1994), although this currently remains untested.

Although there has been no direct dominant negative test of the necessity of a wnt signal as a component of the Nieuwkoop centre signal, expression of a predicted dominant negative allele of XGSK, a homologue of the *Drosophila* gene *zeste white 3/shaggy* which is an antagonist of *wingless* signalling, gives a dorsalised phenotype (Dominguez *et al.*, 1995)

Another recently characterised mesodermal patterning agent, known as noggin, was cloned from a dorsally enriched library made from lithium dorsalised embryos by a functional screen for transcripts with the ability to rescue U.V. ventralised embryos (Smith and Harland, 1992). This secreted factor was subsequently shown, like *Xwnt-8*, to be a potential Nieuwkoop centre mimic/inducer, by its ability to induce a complete secondary axis, including heads, when injected vegetally into ventralised embryos. Maternal expression of *noggin* is lower than levels required to rescue ventralised embryos, and transcripts are uniformly localised during cleavage stages. Consequently it seems unlikely to act endogenously as a Nieuwkoop centre signal. *Noggin* can also rescue ventralised embryos when expressed after MBT and, when added as soluble protein, can dorsalise ventral marginal zone explants (Smith *et al.*, 1993). Unlike activin it retains this latter activity well into the gastrula stage. Not only does noggin have the correct pattering activity to act as the predicted third signal (see fig. 11, page 36) it also has the correct zygotic expression pattern. Expression in the late blastula is localised to the organiser. During gastrulation expression is initially concentrated in the involuting mesoderm and ends up in the presumptive notochord. This obviously makes noggin a prime candidate to be at least a component of the third signal. It is important to note, however, that unlike RNA injection during cleavage, post MBT expression of noggin does not completely rescue ventralised embryos. The induced axes always lack anterior head structures. This may indicate that other components are necessary for complete organiser activity. Alternatively, more complete organiser activity may be dependent on tighter localisation of expression than is afforded by microinjection experiments.

Noggin has a second important activity. As well as being a potential organiser signal it also has the ability to induce cement gland and anterior neural tissue when added to animal cap explants in the form of soluble protein (Lamb *et al.*, 1993).

Finally, another novel putative secreted factor known as *chordin*, which is localised to the organiser and subsequently throughout the dorsal mesoderm during gastrulation and then in the prospective notochord and prechordal plate in the neurula, has recently been shown to have both organiser and (anterior) neural inducing activity (Sasai *et al.*, 1994; Sasai *et al.*, 1995). Chordin is capable of inducing secondary axes, complete from the hindbrain back, and of dorsalising both VMZ's and FGF induced caps. The only known gene with any homology to *chordin* is the *Drosophila* gene short gastrulation (*sog*) (Francois and Bier, 1995)

1.3.3.1. Summary

In summary, and taking the dominant negative receptor data at face value, the molecular evidence seems to back up the three signal model albeit with some additions. Processed Vg-1 is a candidate for both signals 1 and 2 (Nieuwkoop centre signal). Maternal activin A, present as protein imported from the follicle cells during oogenesis, is also a candidate for signal 1. The other major candidate for the Nieuwkoop centre signal is Xwnt-11, with XGSK-1 wild-type and dominant negative phenotypes suggesting that some wnt protein is also necessary for Nieuwkoop centre signalling. The precise nature of the third signal is altogether more confused. Certainly the molecular data supports the idea of separate trunk and head organisers, at least once gastrulation has begun. Noggin is almost certainly a component of the trunk organiser signal, having the right activity and expression pattern, but as it cannot induce anterior head structures when expressed after MBT, it does not seem a likely candidate for the head organiser signal. Rather oddly, considering its broad expression pattern, it is a candidate component of the anterior neural inducing signal. Chordin is probably another trunk organiser signal component, as well as being an anterior neural inducer.

What then of activin, which fits the requirements of a trunk organiser both in terms of dose response, and the changing competence of the tissues 'dorsalised' by the third signal? As activins themselves seem to have been ruled out, the only alternatives seem to be leftover Vg-1 (present equatorially in the egg) or some unidentified activin-like molecule. Factors related to *nodal*, a mouse TGFβ family member essential for gastrulation, which has also recently been shown to have

'organiser activity' by its ability to cause axial duplication in zebrafish embryos, may be possible candidates.

In addition to these three signal model signals, maintenance of both dorsal and ventral mesodermal fate also seems to require signalling. Some form of FGF (or something using its receptor[16]) seems to be required for the maintenance of all mesodermal fates apart from prechordal plate (head). This role can be further subdivided. Some maternal form of FGF is likely to be required, at sub-inducing levels, to act as a competence factor for activin induction of most, but not all, mesodermal markers. Zygotic FGF, probably in the form of XeFGF, is required later in the developmental for the maintenance of posterior/dorsal mesodermal fate. Maintenance of a ventro-lateral fate may involve *Xwnt-8*, and require BMP2 and/or 4 during gastrulation. It also possible that BMP-2 and/or -4 are responsible for competence pre-patterning of the animal cap, and for maintenance of epidermal fate in naive ectoderm.

1.3.4. Postscript - Does D-V patterning of the blastula mesoderm require mesoderm induction after all ?

An intriguing recent paper by Lemaire and Gurdon, questions whether all dorso-ventral patterning of *Xenopus* mesoderm requires mesoderm induction after all (Lemaire and Gurdon, 1994). They stained dorsal and ventral sides of embryos with different vital dyes and then cultured the resulting embryos in calcium and magnesium free medium in order to disrupt cell-cell communication. At stage 6 (32 cell stage) vitelline membranes were removed, the differently labelled cells were manually separated and cultured in a dissociated state until the late blastula when the RNA was harvested. Marker assays for *Xwnt-8* and Gsc, surprisingly showed the same pattern of expression as seen in non-dissociated embryos of the same stage, although *Xbra* expression was shown to be inhibited. Gurdon and Lemaire take this to suggest that cytoplasmic determinants are already in place in the equator of the fertilised egg (post-cortical rotation) to direct the correct dorsal-ventral expression of some mesodermal markers in the blastula marginal zone. This could, presumably be due to some kind of auto-induction, but not by conventional mesoderm inducers which all induce *Xbra* expression.

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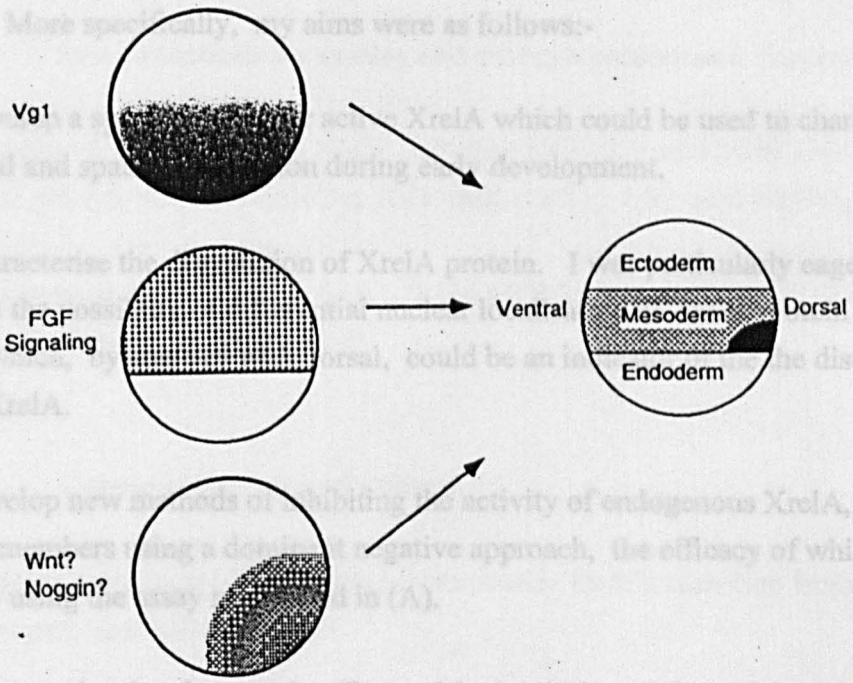
[16]There is intriguing recent evidence for adhesion molecules signalling via FGF receptors (reviewed by Mason, 1994)

FIG. 12:- Model for the role of FGF activin-like, and wnt-like signalling in the pre-MBT Xenopus embryo.



In this model, interactions between FGF and activin-type signalling are proposed to divide the embryo into 3 tissue layers. *Vg1* mRNA is localised to the vegetal hemisphere of the embryo, so its product is most likely to be more concentrated vegetally, with lower levels present in the equatorial zone. FGF signalling is excluded from the vegetal cells and consequently Vg1 signalling in the vegetal hemisphere results in endoderm formation and not mesoderm induction. The Vg1 and FGF signals overlap in the marginal zone, so mesoderm is induced. In the animal caps, Vg1 is absent, and FGF levels are sub-mesoderm inducing, therefore this region becomes ectoderm. Definition of the dorsal ventral axis arises from a dorsally localised signal, which could be maternal Xwnt-11 or noggin, or a very high local level of Vg1. (Model and figure from Cornell *et al.*, 1995).

1.4. Aims and objectives.

The broad aim of the work described in this thesis was to investigate the role(s), if any, of XrelA and other rel family members in the early development of *Xenopus*. More specifically, my aims were as follows:-

(A) To develop a specific assay for active XrelA which could be used to characterise its temporal and spatial distribution during early development.

(B) To characterise the distribution of XrelA protein. I was particularly eager to investigate the possibilty of differential nuclear localisation of XrelA protein in the embryo, which, by analogy with dorsal, could be an indicator of the the distribution of active XrelA.

(C) To develop new methods of inhibiting the activity of endogenous XrelA, and other rel family members using a dominant negative approach, the efficacy of which would be testable using the assay mentioned in (A).

(D) To characterise the phenotypic effects of the inhibition of the activity of XrelA and other rel family members present in embryos, by histology and the analysis of marker expression.

2. Materials and Methods.

2.1. Oocytes, eggs and embryos from *Xenopus laevis.*

2.1.1. Stock solutions for oocyte and embryo culture and fixation.

Barth X medium (BX):- 88 mM NaCl; 1.0 mM KCl; 2.5 mM $NaHCO_3$; 15.0 mM Tris.Cl pH 7.6; 0.3 mM $CaNO_3$; 0.41 mM $CaCl_2$; 0.82 mM $MgSO_4$.

MEMFA (Prepared fresh when needed) :- 0.1M MOPS pH 7.4; 2 mM EGTA; 1 mM $MgSO_4$; 3.7% formaldehyde (filtered).

2.1.2. Oocyte collection and culture.

Oocytes were manually dissected from ovaries freshly dissected from adult female *Xenopus,* and cultured in BX.

2.1.3. Micro-injection of oocytes.

Dissected oocytes were transferred onto a piece of moist filter paper on a microscope slide and injected with approximately 30-40 nl of mRNA (prepared as in section 2.2.16.). Microinjection was carried out under a dissecting microscope using a fine, drawn out glass capillary mounted on a micromanipulator. The needle was linked via a thin gauge pipe to a syringe driven by a vernier, and the system partially filled with oil.

2.1.4. Collection, fertilisation and culture of embryos.

Female *X. laevis* were induced to ovulate by injection of 100 U of follicle stimulating hormone (FSH) 48 hrs to 1 week before laying, and then injection of 600 U of human chorionic gonadotrophin (HCG) 16 hours before laying. Eggs were laid into BX where they remained prior to fertilisation. Eggs were fertilised *in vitro* by brushing with a testis dissected from a male *X. laevis* (stored on ice in BX), in a small volume of BX. After approximately 2 minutes the eggs were flooded with distilled water. Fertilised eggs (as judged by rotation within the vitelline membrane occurring after approximately 20 minutes) were dejellied in 2% cysteine (w/v) pH 8.0. Embryos

were then washed and subsequently cultured in 1/10 BX. Embryos were staged according to Nieuwkoop and Faber (1956).

2.1.5. Micro-injection of embryos.

Embryos were generally injected with mRNA at the two cell stage either bilaterally or unilaterally (as specified) using the apparatus described in 2.1.3.. Prior to injection embryos were transferred to BX containing 5% Ficoll (w/v). After injection, embryos were maintained in Ficoll until stage 6 to reduce leakage. After this stage injected embryos were cultured in 1/10 BX (to avoid exogastrulation).

2.2. Molecular Biology.

2.2.1. Stock solutions for molecular biology.

TE :- 10 mM Tris.Cl pH 7.5; 1 mM EDTA pH 8.0.

TE.1:- 10 mM Tris.Cl pH 7.5; 0.1 mM EDTA pH 8.0.

10 x TBE (pH 8.0):- 108 g/l Tris base; 55 g/l Boric acid; 9.5 g/l EDTA.

20 x SSC:- 3 M NaCl; 0.3M Na Citrate pH 7.0.

10 x C Buffer:- 0.5 M Tris.Cl (pH 7.6); 0.1 M $MgCl_2$; 50 mM Dithiothreitol (DTT); 1 mM spermidine HCl; 1 mM EDTA.

2.2.2. Bacteriological media.

Luria-Bertani medium (LB):- 10 g/l NaCl; 10 g/l bactotryptone; 5 g/l yeast extract.

LB/agar plates:- LB + 15 g/l agar.
Note:- Ampicillin (Amp) was added as required to a final concentration of 100 μg/ml.

NZYCM medium:- 10 g/l NZ amine; 5 g/l NaCl; 5 g/l yeast extract; 1 g/l casamino acids; 2 g/l $MgSO_4.7H_2O$ (pH 7.0).

2.2.3. E.coli strains used:-

(see Sambrook *et al.* (1989) for details)

DH5α
BL21(DE3) - also see Studier and Moffat, 1986.
MC1061
JM101

2.2.4. Transformation of *E.coli* with plasmid DNA.

For Host Strains MC1061 and BL21(DE3):-

E.coli were grown overnight in LB (BL21(DE3) cells were grown in NYZCM) until an optical density of 0.5 was obtained at 600nm ($OD_{600} \approx 0.5$). They were then spun down at 2500 RPM for 5 minutes (4°C), and resuspended in ice cold 0.1 M $MgCl_2$ (half original volume). They were then spun as before and resuspended in ice cold 0.1 M $CaCl_2$ (half original volume) and spun down again before finally resuspending in 1/20th volume of ice cold 0.1 M $CaCl_2$, and incubation on ice for 90 minutes. 100 µl aliquots were combined with an appropriate quantity of plasmid DNA / ligation reaction and incubated on ice for a further 30 minutes. Aliquots were then heat shocked for 2 minutes at 42°C and incubated for a further 10-15 minutes on ice, before plating 10 µl onto one LB / Ampicillin /Agar plate and the remainder onto another. Plates were inverted and incubated overnight at 37°C.

For Host Strain JM101:-

E.coli (JM101) were grown until $OD_{600} \approx 0.4$, spun down (2500 RPM; 5 minutes; 4° C), resuspended in 1/2 volume of 50 mM $CaCl_2$, and incubated on ice for 20 minutes. Cells were then re-pelleted (2500 RPM; 5 minutes; 4°C), resuspended in 1/10th volume of 50 mM $CaCl_2$, and incubated on ice for a further 10 minutes. Appropriate quantities of plasmid DNA/ diluted ligation reaction were added to 200 µl aliquots of cells before incubation on ice for a further 40 minutes, heat shocking and plating as for MC1061.

For Host Strain DH5α:-

The majority of transformations were carried out using frozen stocks of competent DH5α *E.coli*, prepared in the following manner (see Hanahan, 1983).

50 µl of overnight culture were used to seed 25 ml of LB and grown to OD_{550}=0.3-0.4. This was then diluted into two 250 ml flasks of LB and grown to OD_{550} = 0.4-0.5. The flasks were then cooled for 15 minutes on ice and then the cultures spun down in sterile pre-cooled pots (2500 RPM; 10 minutes; 4°C). Pellets were then resuspended in 100 ml ice cold TFB I (0.1 M RbCl; 50 mM $MnCl_2.4H_2O$; 33 mM KAc (pH5.8); 10 mM $CaCl_2.2H_2O$; 20% glycerol (w/v)), and then re-spun (2500 RPM; 10 minutes; 4°C). Pellets were then resuspended in 10 ml (each) TFB II (10 mM MOPS (pH 7.0); 10 mM RbCl; 75 mM $CaCl_2.2H_2O$; 20% glycerol (w/v), separated into 1 ml aliquots and flash frozen in dry ice / methanol. Aliquots were then stored at -70°C until needed.

Aliquots were allowed to thaw on ice when required. 100µl aliquots were used in transformations in the same manner as competent MC1061 cells (see above).

2.2.5. Glycerol stocks.

Glycerol stocks of transformed bacteria were made by adding 0.5 ml of sterile 50% glycerol to 0.5 ml of overnight culture, vortexing vigorously to mix, and flash freezing on dry ice / methanol. Stocks were then stored at -70°C. Stabs were plated from these stocks as required.

2.2.6. Large scale plasmid isolation.

'Ice Prep' Method

100 ml of overnight culture of the appropriate transformed *E.coli* (in LB + Ampicillin 100 µg / ml) was centrifuged (3000 RPM, 10 minutes), and resuspended in lysis buffer (50 mM glucose; 25 mM Tris pH8.0; 10 mM EDTA). The bacteria were then lysed by the addition of 8 ml of alkaline SDS (0.2 M NaOH; 1% SDS), followed by gentle inversion (to avoid shearing of genomic DNA), and incubation on ice for 5 minutes. The lysate was neutralised by the addition of 6 ml 3M KAc pH 4.8), followed by inversion to mix, and incubation on ice for a further 10 minutes. The resulting precipitate was removed by centrifugation at 8000 RPM for 15 minutes, and the remaining nucleic acids were precipitated by the addition of 17 ml of isopropanol, followed by 10 minutes incubation at -70°C. After centrifugation at 8000 RPM for 20 minutes (4°C), the air dried precipitate was resuspended in 2 ml of TE.1. This solution was then further purified by the addition of 2.5 ml of 4 M LiCl and incubation on ice for 60 minutes. After removal of the precipitate by centrifugation at 8000 RPM for 10 minutes (4°C), the nucleic acids were again precipitated by the addition of 10 ml of ethanol followed by incubation on ice for 10 minutes and centrifugation at 8000 RPM for 15 minutes. RNA was removed by resuspending the pellet in 400 µl of TE.1 containing 25 µg/ml RNase A, followed by incubation at 37°C for 10 minutes. After the addition of 20µl 10% SDS and heating to 70°C for 10 minutes the solution was extracted once with phenol, once with phenol / chloroform / isoamyl alcohol (100:96:4) (P:C:I), and once with chloroform. Plasmid DNA was precipitated by the addition of 1/10th volume of 3M NaAc (pH 6.8) and 2.5 volumes of ethanol and incubation at -20°C for 30 minutes. The resulting pellet was washed with ice cold 70% ethanol, dried in air, and resuspended in TE. The yield was determined spectrophotometrically (OD_{260nm})

CsCl gradients (plasmids for injection into embryos)

Although the Ice Prep method yielded plasmid pure enough for most purposes, it was not suitable for preparation of reporter constructs for direct injection into *Xenopus* embryos, due to toxicity. DNA for running on the gradients was prepared using the first three steps of the ice prep method. Following precipitation with isopropanol, pellets were dried in a vacuum desiccator and resuspended in 20 ml of TE (pH 8.0). 20 g of CsCl was then added and dissolved by vortexing, along with 600 µl ethidium bromide (EtBr) (10 mg / ml) for visualisation of nucleic acids under ultra violet (UV) irradiation. Solutions were then loaded into sealable ultracentifuge tubes, covered with a layer of heavy white paraffin, and balanced to within 10 mg before sealing. These tubes were then ultracentrifuged under a vacuum at 45 000 RPM for 18hrs and allowed to decelerate without breaking. DNA was then visualised under UV and the lower band (supercoiled plasmid) removed using a hypodermic needle and syringe, avoiding any precipitate on the side of the tube. EtBr was removed by extraction into butanol (4 times with 1 volume), and CsCl removed by overnight dialysis against TE (pH 8.0) at 4°C (2 changes 1 L, 1hr each, then 1 L overnight). Finally preps were extracted with phenol, P:C:I, and chloroform before precipitation with NaAc and ethanol (as in previous method). Plasmids injected in circularised form were precipitated in the same manner twice more before being washed twice with ice cold 70% ethanol, and resuspension in millipure water. Plasmids requiring linearisation prior to injection were digested as appropriate after the first precipitation, and then cleaned again by extraction with P:C:I, chloroform, and then precipitated as for circular plasmid.

2.2.7. Small scale isolation of plasmids ('mini-preps').

Small scale plasmid isolation was carried out using essentially the same initial steps as in the ice prep. but scaled down. 1 ml of overnight culture was spun down (13 000 RPM; 1 minute; room temperature (RT)) and resuspended in 100 µl lysis buffer, lysed by the addition of 200 µl alkaline SDS (mixing by gentle inversion), and incubation on ice for 5 minutes, and neutralised with 150µl 3M KAc pH 4.8, with a further incubation on ice of 10 minutes. After centrifugation at 13 000 RPM for 5 minutes the supernatant was extracted with 1 volume of P:C:I and then with 1 volume of chloroform, before precipitation of nucleic acids by the addition of 2.5 volumes (≈1 ml) of ethanol and incubation at -20°C for 30 minutes. After centrifugation at 13K RPM for 20 minutes pellets were resuspended in 30 µl TE containing RNase A (25 µg / ml)

2.2.8. Agarose gel electrophoresis of DNA and RNA.

Agarose gels of the appropriate percentage were made with 0.5 x TBE containing 0.5 μg/ml EtBr were run in 0.5 x TBE (+ 0.5 μg/ml EtBr) at 100V. For RNA gels tanks were pre-treated with 6% H_2O_2 for 1 hr prior to use and all glassware used for the preparation of buffers and the gel was pre-baked. DNA was first mixed 6:1 with 6 x agarose gel loading buffer (3 x TBE; 0.5% bromophenol blue; 0.5% xylene cyanol; 10% glycerol) before loading onto gels.

2.2.9. Elution of DNA from agarose gel slice.

Appropriate bands were visualised using UV trans-illumination and excised from agarose gels using a razor blade with the minimum UV exposure possible, to minimise nicking. All purification of DNA from agarose gel slices was carried out using a Geneclean II kit (Bio 101).

2.2.10. Restriction digests.

All restriction digests were carried out in commercially supplied buffers at manufacturers recommended conditions with a minimum dilution of enzyme stock of 1/10.

2.2.11. Dephosphorylation of linearised vector ligatable ends.

Dephosphorylation reactions were carried out using calf intestinal alkaline phosphatase (Gibco-BRL) in C-buffer (see section 2.2.1.)

2.2.12. Ligations.

All ligations were carried out with T4 DNA ligase (Gibco-BRL) in the commercially supplied buffer, usually for 4 hours at 18°C (for sticky ended ligations), or from 8 hrs to overnight at 4°C for blunted ended ligations. Ligation mixes were always diluted 1 in 5 into sterile distilled water prior to transformation, in order to dilute out the polyethylene glycol (PEG) in the ligation buffer.

2.2.13. Preparation of oligonucleotide stocks.

All oligonucleotide (oligo) stocks were prepared by taking 100 µl from a lyophilised master stock resuspended in 500 µl TE and extracting with phenol and chloroform. Oligos were then precipitated by adding 9 volumes of butan-1-ol and vortexing rapidly. After centrifugation (20 minutes; 13 000 RPM), pellets were washed with ice cold 70% ethanol and allowed to dry briefly in air before resuspending in 100 µl sterile TE.

2.2.14. Polymerase Chain Reaction (PCR).

All PCR primer pairs were designed using PRIMER DESIGNER (Scientific and Educational Software) to optimise (equalise) melting temperatures and avoid the risk of primer dimer and secondary structure formation which might prevent proper priming. PCR reactions were generally carried out using the following conditions:-

For a 100 µl reaction (reactions were frequently scaled down to 50 or 20 µl):-

10 µl 10x Taq buffer
x µl 50 mM Mg Cl_2 ($MgCl_2$ concentration was usually titrated from 1 to 2.5 mM, and the lowest successful concentration used for preparative PCR reactions to reduce error incorporation (Innis *et al.*, 1990)).
y µl nucleotide mix (Usually to give a final concentration of 100 µM, although lower concentrations were sometimes used to improve fidelity in preparative PCR reactions (Innis *et al.*, 1990)).
z µl target DNA (If target concentration was known then generally 100 pg to 1 ng /100 µl reaction was used, but higher concentrations were sometimes empirically found to be necessary).
0.5 µl Taq DNA polymerase (5 U/µl)
This was made up to 100 µl with millipure water, and overlaid with UV treated light paraffin (UV treatment destroys any contaminating nucleic acids, e.g.:- from pipette aerosols).

PCRs using plasmid as target were preceded by a hot start (3 minutes at 95°C) prior to adding the enzyme to melt the target sufficiently. Where it was found to be necessary, plasmid targets were linearised. The conditions for the PCR reaction cycling were determined as follows:-

Melting:- 15 seconds at 95°C.
Annealing:- 30 seconds at 1-2°C below melting temperature predicted by PRIMER DESIGNER, or determined empirically if necessary.
Extension:- 45 seconds, plus a further 30 seconds/kb product length for each extra kb over 2 kb, at 72°C.

2.2.15. Colony screening by PCR.

Colonies were picked with a sterile pipette tip into 1 ml of sterile distilled water, which was then vortexed thoroughly. A loop-full of the resulting suspension was then streaked onto a segment of an LB/Amp agar plate, and the remainder boiled for 5 minutes. 5 µl of dilute lysate was used per 50 µl diagnostic PCR.

2.2.16. Transcription and capping of synthetic mRNA.

Template preparation

Templates were prepared from Ice Prep. DNA by digestion to completion with the appropriate restriction enzyme, followed by digestion for at 56°C for 30 minutes with proteinase K (100 µg/ml) in the presence of SDS (0.5 %) and EDTA (5 mM). Following this preparations were further purified by extractions with phenol, P:C:I and chloroform, before precipitation with 1/10th volume of NaAc pH 6.8 and 2.5 volumes of ethanol. Resulting pellets after 20 minutes centrifugation at 13 000 RPM were washed with ice cold 70% ethanol and resuspended in sterile TE made with millipure water.

Note :- It is important not to gel purify templates, as nicking resulting from visualisation with UV can have an adverse effect on transcription efficiency.

Transcription

Transcriptions were carried out using a mMessage mMachine *in vitro* transcription kit (Ambion). This system uses a ratio of rGTP to CAP analogue of 4:1, rather than the more conventionally used 10:1. Although this leads to a reduction in the efficiency of capping of around 20%, it leads to an increase in yields of around 3-5 fold. After 3-4 hrs template was removed by digestion with DNase I (2 U) for 15 minutes at 37°C, and the RNA was cleaned and precipitated three times with ethanol

and ammonium acetate, washed twice with 70% ethanol and resuspended in millipure water for injection into oocytes or embryos.

RNA yields were calculated by measuring OD_{260nm} and batches of RNA were tested for equivalent levels of translation as in 2.2.17. Groups of transcriptions carried out using a common stock were generally found to give approximately equivalent tranlation levels as judged by this assay.

2.2.17. *In vivo* translation assays.

Groups of 10-12 oocytes were injected with the appropriate mRNA (as in 2.1.3.) and incubated overnight in BX containing 5-10 μCi of ^{35}S-methionine (1000 Ci/mmol). Oocytes were subsequently homogenised as in section 2.3.1., and products were visualised by SDS-PAGE, as in section 2.3.4.

2.2.18. Whole mount *in situ* hybridisation.

(Method from Richard Harland, modified by Derek Gatherer)

Probe synthesis

Templates were prepared as for transcription of synthetic mRNA (see section 2.2.16.). Transcriptions were carried out with the appropriate RNA polymerase (50 U) to produce antisense RNA (see Appendix 2). Transcription / labelling reactions using Digoxygenin-11-UTP (DIG-UTP; Boehringer) contained the following :- 0.5-1 μg template; 0.25 mM DIG-UTP; 0.5 mM UTP; 0.75 mM each ATP, CTP, and GTP; 10 U RNasin; 4 μl 5 x transcription buffer (Gibco-BRL) - made up to 20 μl with millipure water. Probes were then treated with 2 U DNase I for 15 minutes at 37 °C and then precipitated once with NaAc and ethanol, washed with ice cold 70% ethanol and resuspended in 50 μl millipure water. Probe concentrations were estimated spectrophotometrically (OD_{260}) and stored at -70°C until needed.

Embryo fixation

Embryos of the appropriate stages were fixed overnight in freshly made MEMFA at 4°C with the vitelline membranes removed (membranes were manually dissected shortly after placing the embryos in fixative). Embryos were stored at -20°C under 100% ethanol (after dehydration through increasing concentrations of ethanol).

Pre-hybridisation Washes

Embryos were re-hydrated through a graded series of ethanol concentrations into PTw (PBS + 0.1% Tween-20 (SIGMA)). They were then washed three times for 5 minutes each in PTw, before the addition of PTw containing 1-5 µg/ ml proteinase K and incubation at room temperature for 5 minutes. Note:- for pre-neurula stages low concentrations of proteinase K were used, and the embryos were observed under a dissecting microscope for signs of damage during incubation. After proteinase digestion embryos were washed 3 times for 5 minutes with PTw and then re-fixed in MEMFA for 20 minutes (embryos showing damage during digestion were fixed immediately). Embryos were then washed a further 4 times (5 minutes) with PTw to remove fix and then transferred to 1:1 PTw:Hyb mix (se below) for 5 minutes before transfer to full strength Hyb mix (50% Formamide (FLUKA); 5 x SSC; 100 µg/ml heparin (SIGMA); 1 mg/ml yeast torula RNA (SIGMA); 0.1% Tween-20; 0.1% Chaps (SIGMA); 2% Boerhinger blocking powder) at 60 °C for 5-6 hours.
(Note:- Baked glass vials were used for this and subsequent stages.)

Hybridisation

Embryos were placed in fresh hyb. mix containing 1 µg/ml of probe at 53°C overnight.

Post-hybridisation washes and RNase A digestion.

Hyb mix plus probe was replaced with fresh hyb mix preheated to 53°C, and the embryos were further heated to 60°C. The embryos were then washed at 60°C with preheated washes as follows:-
Once with 50% Hyb: 50% 2xSSC, 0.3% CHAPS; 10 minutes
Once with 25% Hyb: 75% 2xSSC, 0.3% CHAPS; 10 minutes
Twice with 2xSSC, 0.3% CHAPS; 20 minutes each

Embryos were then transferred to RNase digestion buffer (2 x SSC; 0.3% CHAPS; 20 µg/ml RNase A) and incubated at 37°C for 30 minutes. Following this treatment the embryos were washed as follows:-
Twice with 2 x SSC, 0.3% CHAPS, at RT for 10 minutes
Twice with 0.2 x SSC, 0.3% CHAPS at 60°C for 30 minutes
Twice with PTw + 0.3% CHAPS at 60°C for 10 minutes

Antibody Incubation

Embryos were transferred to antibody blocking solution (PTw; 0.1% CHAPS; 0.5% Boehringer blocking powder) and left to equilibrate for 5 minutes before replacing with fresh antibody blocking solution and incubating at 4°C for 4 hrs. After blocking embryos were transferred to fresh antibody blocking solution containing a 1/2000 dilution of alkaline phosphatase linked anti-digoxygenin antibody (Boerhinger), and incubated overnight at 4°C.

Colour reaction

Embryos were washed 4 times for 60 minutes each with PTw containing the phosphatase inhibitor Levamisol, 5 mM (SIGMA), and then transferred to chromogenic buffer (100 mM Tris.Cl pH 9.5; 50 mM $MgCl_2$; 100 mM NaCl; 0.1% Tween-20; 5 mM Levamisol) in a multi-well plate. After equilibration for 5 minutes this was replaced with fresh chromogenic buffer containing 4.5 µl/ml NBT (from 75 mg/ml stock in 70% dimethylformamide (DMF)) and 3.5 µl/ml BCIP (from 50 mg/ml stock in 100% DMF). When desired signal to background ratio was reached (anywhere from 4 hrs to overnight) the reaction was quenched by washing for 5 minutes with TE pH 8.0, fixing for 20 minutes in MEMFA and then dehydrating in ethanol for storage (at 4°C) prior to photography under a dissecting microscope.

2.3. Protein extraction, separation, purification and detection.

2.3.1. Protein extraction from oocytes and embryos.

Protein extracts for gel mobility shift assays (GMSA) were made by homogenisation of embryos or oocytes using a glass homogeniser into Buffer A (10 mM Hepes pH 8.0; 50 mM NaCl; 0.5 M Sucrose; 1 mM EDTA; 0.5 mM spermidine; 0.15 mM spermine; 0.2% Triton X-100; 7 mM β-mercaptoethanol; 15% glycerol) with the addition of protease inhibitors PMSF (1 mM), leupeptin (0.5 μg/ml), and aprotinin (0.5 μg/ml). After centrifugation to remove yolk, and other insoluble material (13 000 RPM; 5 minutes; 4°C), the extract was flash frozen on dry ice.

Oocyte nuclear extracts for GMSA's were made by homogenisation of manually dissected nuclei into buffer B (20mM TrisCl pH 79; 1.5mM $MgCl_2$; 500mM KCl; 5 mM NaF; 1 mM Na_3VO_4; 20% glycerol) with the addition of DTT (to 0.5 mM) and protease inhibitors (as above) immediately prior to extraction, at 10 μ l/nucleus. These extracts were then concentrated using Centricon-10 tubes as in the manufacturers instructions which reduced the total volume by approximately five fold.

Protein extracts for SDS PAGE electrophoresis (for Western blotting) were made by the same method with Barth X (+ protease inhibitors) being substituted for buffer A. After centrifugation the cleared lysate was mixed with 0.8 volumes SDS Page loading buffer (see section 2.3.4.) and 0.2 volumes 1M DTT and boiled for 5 minutes immediately prior to loading.

2.3.2. Extraction of protein from E.coli.

Bacterial cultures were pelleted by centrifugation (5 minutes 10000 RPM), and re-suspended in 50 μl of ice cold PBS containing a cocktail of protease inhibitors (PMSF (1 mM), leupeptin (0.5 μg/ml), and aprotinin (0.5 μg/ml) per ml of culture.

For quick testing of expression suspensions were mixed with 0.8 volumes 2x SDS-PAGE loading buffer (125 mM Tris.Cl; 5% SDS; 0.2% bromophenol blue; 25% gycerol) and 0.2 volumes of 1 M DTT. Genomic DNA was sheared by rapid pipeting through a 200μl tip and the mixture was boiled for 5 minutes before loading on an SDS-PAGE gel.

For preparation of fusion proteins for use as antigen, or affinity purification bacteria re-suspended in PBS were lysed by sonication. This step also serves to shear genomic DNA. Insoluble material was removed from the lysate by centrifugation (5 minutes; 13000 RPM; 4°C). Cleared lysate produced in this fashion was used for affinity purification of GST fusion. Cleared lysate containing fusion protein for purification by electroelution was mixed with 0.8 volumes of 2 x SDS-PAGE loading buffer (see above) and 0.2 volumes of 1 M DTT and boiled for five minutes before running on a 3 mm thick SDS-PAGE gel

2.3.3. Protein Assays.

All protein assays were carried out using a Bio-Rad protein assay kit according to the manufacturers instructions.

2.3.4. Discontinuous SDS polyacrylamide gels.

Acrylamide mix at 30% (29:1 acrylamide : N, N'-methylenebisacrylamide) was used to make discontinuous gels for protein separation. The concentration of acryamide in the separating gel was varied according to the protein sizes to allow the best resolution of the proteins, using the following as a guide:

Acrylamide (%)	linear range (KDa)
15	12-43
10	16-68
7.5	36-94
5	57-212

From Sambrook *et al.*, 1989.

As an example: 10% separating gels were made using 10% acrylamide mix; 375 mM Tris.Cl (pH 8.8); 0.1% SDS; 0.1% ammonium peroxodisulphate (APS) and 0.04% TEMED (N,N,N',N'-tetramethylethylenediamine). The gels were poured leaving enough space for a 0.5-1 cm stacking gel and overlayed with a small amount of butan-1-ol until set. The butan-1-ol was rinsed off and a 5% stacking gel (5% acrylamide; 125 mM Tris.Cl (pH 6.8); 0.1% SDS; 0.1% APS; 0.1% TEMED) poured before inserting a comb and allowing to set. Gels were run in Tris-glycine - SDS running buffer (25mM Tris; 250mM glycine (pH 8.3); 0.1% SDS) at 100 Volts through the stacking gel and subsequently at 200 volts.

2.3.5. Staining with coomassie blue.

Proteins were visualised by staining the gels in 5 volumes of coomassie blue stain (2.5% coomassie blue; 45% methanol (v/v); 10% glacial acetic acid.) for 4 hours at RT. Gels were then destained with 3-4 changes of destain (45% methanol; 10% glacial acetic acid) for 4-8 hours. Gels were stored dried.

2.3.6. Electroelution of protein from an SDS-PAGE gel slice.

SDS-PAGE gels were run as in 2.3.4.and stained without fixing in an aqueous solution of coomassie blue (1%). A slice corresponding to the desired size of band was then excised with a razor blade and placed in a sealed piece of dialysis tubing along with 10 volumes of electro-elution buffer (0.2 mM Tris acetate pH 7.4; 100 mM DTT; 0.1% SDS). This was then arranged across the bottom of an agarose gel tank filled with enough electro-elution running buffer (50 mM Tris acetate pH 7.4) to just cover the dialysis tubing and run at 100V for 4 hrs, or until there was no visible coomassie staining left in the slice.

2.3.7. Removal of SDS from electro-eluted protein.

To the eluate was added SDS (to give a final concentration of 1%) and KAc (to give a final concentraion of 0.3 M). This mixture was then incubated on ice for 30 minutes before being spun at 10 000 RPM for 30 minutes at 0°C. The resulting pellet was washed twice with ice cold acetone / HCl (0.1 M) before being resupended in 1 ml of ice cold 10% trichloro-acetic acid (TCA) and centrifuged again (10 000 RPM; 30 minutes; 0°C). This time the pellet was washed twice with ice cold acetone, dried and resupended in 0.5 ml sterile PBS.

2.3.8. Western blotting.

SDS-PAGE gels were blotted onto nitrocellulose filters (Hybond C (Amersham)) using Gibco-BRL electroblotting apparatus according to the manufacturer's instructions, in a transfer buffer consisting of (39 mM glycine; 48 mM Tris base; 0.1% SDS, 20% methanol (v/v). Transfer was tested by staining the filter with 0.2% Ponceau S (Sigma) in 3% TCA, which was then removed by washing in PBS.

Blocking and antibody incubation of western blots

Blocking and subsequent antibody hybridisations were carried out in blotto (5% Marvel; 0.02% Na azide in PBS). Filters were pre-blocked for 1 hr at RT. Primary antibody hybridisation was usually carried out overnight at 4°C (with shaking), filters were then washed 4 times 5 minutes with shaking in PBS, before hybridising with the horseradish peroxidase (HRP) linked secondary antibody at RT, usually for a further 2 hrs. Filters were washed a further 4 times with PBS at RT prior to staining for (HRP) activity using diaminobenzidine (DAB).

Detection of antibody linked HRP activity with DAB

Filters were stained with a 0.6 mg/ ml solution of DAB in 50 mM Tris.Cl pH 7.6 with 1 µl/ ml of 30% H_2O_2 added immediately prior to use. Staining was allowed to proceed until the desired signal to background ratio was achieved, and the reaction quenched by washing thoroughly with PBS. In the case of particularly weak signals staining was enhanced by the addition of 1/10th volume of 0.3% $CoCl_2$ to the staining solution.

2.3.9. Whole-mount immunohistochemistry.

Fixation and storage

Embryos were fixed in fresh MEMFA for 2 hours at room temperature before dehydrating through an ethanol series and stored at -20°C in 100% ethanol. Immediately before use they were gradually rehydrated through an ethanol series made up with PBS (90%, 80%, 70%, 50%, 25%), and then allowed to equilibrate in PBS for 5 minutes.

Blocking, washing, and hybridisation.

Embryos were blocked for 1 hour in PBT (PBS + 2 mg.ml BSA (fraction V) + 0.1% Triton X-100) + 10% goat serum, with shaking at room temperature. Primary antibody incubations were carried out in PBT + 10% goats serum containing the appropriate dilution of primary antiserum, overnight at 4°C with shaking. Embryos were then washed 4 times with PBT at room temperature, with shaking. In some cases, to reduce background a further overnight wash was carried out at 4°C, with shaking. Secondary antibody incubations were carried out in PBT + 10% goat serum

containing the approproate dilution of secondary antibody. Embryos were then washed as for after primary incubation.

Confocal microscopy

Flourescent staining of embryos was visualised by optical sectioning using a confocal microscope. Images were obtained by video printing.

2.3.10. Detection of β-galactosidase activity in whole embryos.

Embryos were washed in PBS, and fixed in X-gal fix (2% formaldehyde; 0.2% glutaraldehyde; 0.02% Nonidet P40 (BDH); 0.01% sodium deoxycholate in PBS) for 1 hour at RT. After washing 3 times with PBS (5 minutes each) embryos were transfered to X-gal stain (5 mM potassium ferricyanide; 5 mM potassium ferrocyanide; 0.1% X-gal; 2 mM $MgCl_2$ in PBS), and gently nutate in the dark until staining was clearly visible (usually 3-4 hrs). Stained embryos were then re-fixed in MEMFA overnight, and stored in 100% ethanol. X-gal stained embryos to be used subsequently for *in situ* hybridisation were stored under ethanol at -20°C until needed.

2.3.11. Chloramphenicol acetyl transferase (CAT) assays.

Embryos were homogenised into ice cold Tris.Cl pH 7.5 (50 µl/embryo) and centrifuged for 5 minutes. The supernatant was stored at -70°C until needed. Extracts were assayed for soluble protein as in section 2.3.3.

For each assay 50 µl of extract was added to 5 µl ^{14}C-chloramphenicol (25 µCi/ µl; 98 µCi/mmol) diluted to 45 µl with sterile distilled water. This mixture was then warmed to 37°C and the reaction started by the addition of 5 µl Acetyl Coenzyme A. After incubation at 37°C for 60 minutes the reaction was quenched by the addition of 90 µl of ice cold 0.25 M Tris.Cl (pH 7.5) and extraction with 1 ml of ice cold ethyl acetate (with vigorous vortexing). After centrifugation at 13 000 RPM for 5 minutes the upper phase was removed and dried under a vacuum. Pellets were resuspended in 20 µl ethyl acetate and spotted onto a thin layer chromatography plate (TLC aluminium sheets, silica gel 60 (MERK)). The plate was run in a pre-saturated tank containing 150 mls chloroform:methanol (19:1).

The assay product was visualised using a Molecular dynamics phosphorimager and quantified using Image Quant software

2.4. Detection of DNA binding activity (Gel Mobility Shift Assays (GMSA)).

(This method was a personal communication from Laura Sanz (Ctr Biol Molec, Csic, Univ Autonoma Madrid, Madrid 34, Spain.)

2.4.1. Annealing oligonucleotides for probes/competition.

Oligonucleotide stocks prepared as in section 2.2.13. were mixed in TE to give a final concentration of 2 ng/µl. This mixture was then heated to 98°C in a water bath which was then allowed to cool to room temperature. Stocks were stored at -20°C.

2.4.2. Labelling the probe.

Probe labelling reactions consisted of 10ng of annealed oligonucleotide., 40 µCi ^{32}P-γ -ATP (5000 Ci/ mmol), 1.5 µl 10 x C-buffer, and 1 µl T4 polynucleotide kinase (PNK) made up to 15 µl with sterile distilled water (SDW). Reactions were incubated for 60 minutes at 37°C and then extracted with P:C:I and chloroform before precipitating with Na Ac and ethanol (as in Ice Prep method) with the addition of 1 µg tRNA as carrier. Pellets were washed once in ice cold 70% ethanol and resuspended in 100 µl TE.

2.4.3. Binding reaction.

Binding reactions consisted of 0-10 µl of protein extract, prepared as in section 2.3.1., made up to 10 µl with buffer A (section 2.3.1.) if necessary, with 6 µl 5 x binding buffer (20% glycerol; 5 mM $MgCl_2$; 2.5 mM EDTA; 2.5 mM DTT; 250 mM NaCl; 50 mM Tris.Cl pH7.5; poly (dI-dC-dI-dC) (SIGMA) 0.5 µg/µl, or as titrated. Cold wild type or mutant competitor was added as required, and the reaction made up to 29 µl and incubated at RT for 5 minutes prior to adding 1 µl of probe (prepared as above) and incubating for a further 5-10 minutes at RT.

2.4.4. Electrophoresis.

Samples were run on a non-denaturing 6% polyacrylamide gel (acrylamide : bisacrylamide 29:1) in 1/4 x TBE at 200 V for 2 hrs in a cold room (4° C).

2.5. Histological Examination of Paraffin Embedded Tissue.

2.5.1. Fixation and embedding.

Samples were fixed overnight in MEMFA and dehydrated with an ethanol series (25%, 50%, 70%). Long term storage of samples was in 70% ethanol at 4°C. Prior to embedding embryos were further dehydrated into absolute ethanol, again through a graded series (80%, 90%, absolute, absolute), and then cleared using Histoclear (National Diagnostics). They were then taken through a further change of Histoclear and one change of Histoclear/wax (Histoplast) (1:1) at 60°C, followed by 3 changes of wax (1 hour each, at 60°C). Embryos were then embedded in fresh wax in plastic molds.

2.5.2. Sectioning.

Trimmed blocks containing the embedded embryos were cut into 10μm sections on a Bright microtome using disposable blades (Raymond Lamb). Ribbons of sections were floated in a water bath at 45°C, and then dried overnight (60°C slide drier) onto slides.

2.5.3. Staining and mounting.

Sections were dewaxed by washing twice in Histoclear (15 minutes each), and then rehydrated through a graded ethanol series (100%, 95%, 80%, 70%, 50%, 30%, 25%). Rehydrated sections were stained in Haematoxylin solution (SIGMA) for 4 minutes, rinsed in tap water for 5 minutes, and then counter-stained with 1% Eosin (BDH) for 1 minute and rinsed in tap water for a further minute. In preparation for mounting sections were rehydrated through the ethanol series detailed above, and then taken through two changes of Histoclear before mounting under coverslips using Depex mountant (BDH).

3. Detecting XrelA protein.

3.1. Introduction

An antiserum which recognises XrelA would be useful for a number of reasons. By analogy with *dorsal* in *Drosophila*, and also with other rel family members, differential activation of an XrelA containing complex might be expected to exhibit itself as differential nuclear localisation of that complex. The detection of endogenous XrelA protein in whole or sectioned embryos by immunohistochemistry might therefore provide a means of mapping activation of the endogenous protein in embryos. Another potential use of the antiserum would be to identify DNA binding complexes containing XrelA detected in gel mobility shift assays (see page 83).

During the course of this work the distribution of XrelA was mapped by another group, first by using anti v-rel antiserum, which was shown to cross react with XrelA in western blots, and then later with antiserum raised against XrelA itself (Bearer, 1994). After the publication of this work I decided to concentrate my efforts elsewhere, rather than attempt to repeat the results using my antiserum. However, as the antiserum raised was used in GMSA supershift assays (see page 83), I have detailed how it was raised in this chapter.

Bearer found that in the fertilised egg the protein is concentrated mainly in the nuclear region. However, by stage 4 (8 cells) the protein is arranged in a distinct animal to vegetal gradient in the cytoplasm. This may be just an effect of dilution of the cytoplasm by yolk in the vegetal hemisphere; a general effect born out by the fact that soluble protein yields from the vegetal hemisphere are comparatively low compared to those from other regions (H.R. Woodland, personal communication). Nuclear localisation is not seen until around about stage 7½, when it occurs in the nuclei of animal cap and equatorial cells. Nuclear staining becomes more intense the closer nuclei are to the animal pole. No staining of nuclei in the vegetal hemisphere is seen at this or later stages, but rather staining appeared to be localised in these cells to (unidentified) perinuclear particles. Nuclear staining in the animal hemisphere is still apparent at stage 10, but decreases to undetectable levels during gastrulation. The possible implications of these data, which are obviously an important consideration for any models of the role of XrelA in early development, are discussed later (see chapter 8.)

3.2. Results

3.2.1. Purification of protein for raising antiserum.

In order to raise a polyclonal antiserum against XrelA it was important to be able to produce milligram quantities of relatively pure XrelA protein for use as an antigen. One way of doing this is to use a bacterial expression vector designed for producing affinity purifiable fusion proteins. One such group of vectors, known as pGEX vectors, allow the production of proteins fused to glutathione-S-transferase (GST), which can be inducibly expressed in bacteria (Smith and Johnson, 1988). The fusion protein, if soluble, can then be purified from a cleared lysate of the expressing bacteria using glutathione bound to agarose beads. The affinity purified protein can be eluted from the beads using soluble glutathione, and finally cleaved to separate the protein from the fused GST using a protease, a cleavage site for which has been incorporated at the fusion point. This approach, if successful, can be very powerful but problems can occur if the fusion product is insoluble or toxic.

With the aim of producing an XrelA/GST fusion protein expression construct, the open reading frame of XrelA was cloned into the fusion expression vector pGEX-*2T* (see Appendix 1). This vector allowed easy cloning of an in frame fusion. This was done by subcloning from the transcription construct 6b (Richardson, 1991; see Appendix 1), by digesting with BamHI and BglII, and cloning the resulting fragment into pGEX-*2T* digested with BglII. Clones containing insert in the desired orientation (from here referred to as pGEX-*2TXr*), as tested by diagnostic restriction digests, were transformed into *E.coli* JM101. Cultures of these cells were tested for the expression of fusion protein at various time points after induction with IPTG (cultures were induced when the density reached OD_{600}= 0.8) by the rapid testing method decribed in section 2.3.2.. Unfortunately, and for whatever reason, levels of fusion protein in extracts from pGEX-*2TXr*-transformed *E.Coli* induced with IPTG, were undetectably low (see figure 13), even after subsequent affinity purification with glutathione-agarose beads (data not shown). This was also true of extracts made soon after induction (data not shown), suggesting the fusion product was either highly unstable or toxic. This made the purification of enough protein for raising the antiserum impractical.

As an alternative to the pGEX system, it was decided to use an already existing inducible fusion protein construct in the form of a pET fusion construct made by Jill Richardson (Richardson, 1991). This construct, known as pET-*3a526*,

consists of the complete open reading frame (ORF) of XrelA sub-cloned (in frame) into the fusion site of pET-*3a* (see Appendix 1 for map). pET-3 vectors are a series of high level expression vectors which allow the production of proteins fused to the first 10 amino acids of T7 *gene 10*, under the control of a T7 RNA polymerase promoter. Expression can be controlled when the construct is transformed into the *E.coli* lysogen strain BL21 (DE3), which has an integrated phage lambda derivative containing the T7 RNA polymerase gene under the control of the *lacZ* promoter, making it inducible by IPTG (Studier and Moffatt, 1986). Because basal (uninduced) transcription from the T7 promoter in this system is negligible by comparison with systems which use direct control of expression by the *lacZ* promoter, it allows the production of toxic gene products as long as the culture has enough time to grow prior to induction. The main problem with using such a system is of course that it does not allow easy subsequent purification. The best approach seemed to be to gel purify the fusion protein and the isolate it by electroelution. As native and fixed protein were the intended targets of the antiserum, it was decided to further purify the eluate to remove SDS. The method chosen (see section 2.3.7.) involves a trichloroacetic acid (TCA) preciptation step, which is not necessarily disadvantageous as preciptated protein is often highly antigenic (Harlow and Lane, 1988).

Protein was extracted from *E.coli* BL21(DE3) transformed with pET-*3a526* and induced for 3 hours with 0.1 mM IPTG, as described in section 2.3.2. Extracts were run on a 3 mm thick SDS-PAGE gel as decribed in section 2.3.4., which was then lightly stained with Coomassie blue (1% in distilled water). The induced band was then excised and electroeluted as described in section 2.3.6., and the eluate treated to remove SDS as described in section 2.3.7., and resuspended in sterile PBS for injection. Yields were assayed using Bio-Rad Protein assay kit as described in section 2.3.3. Preparations were stored in aliquots at -70°C until required. Induced fusion protein and the final purified product can be seen on the SDS-page gel shown in figure 14.

3.2.2. Raising and testing the antiserum.

For the primary injections, the protein extract described in the previous section was mixed with an equal volume of Freund's adjuvent and injected intramuscularly into two rabbits (~30 µg / rabbit). Rabbits were then boosted every two weeks by intravenous injection of the protein extract without adjuvent (~15 µg / rabbit). Small test bleeds of around 5-10 mls were taken from injected rabbits prior to each boost and serum was prepared by decanting from clotted blood and stored in aliquots at -20°C.

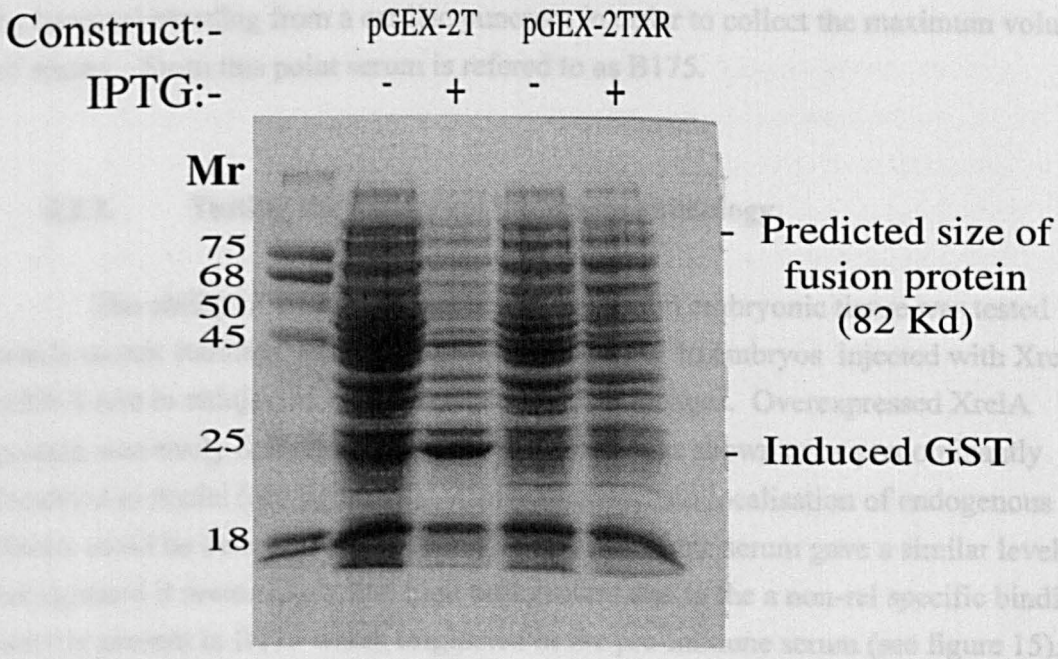
FIG. 13:- pGEX-2TXR does not express detectable levels of XrelA/GST fusion protein.

Cultures of *E.coli* JM101 transformed with pGEX-2T and the XrelA/GST fusion construct pGEX-2TXR were grown to OD_{600}= 0.8 and then induced to express GST / GST-XrelA fusion protein by the addition of IPTG to give a concentration in the culture of 0.1mM. After a 1hour incubation protein extracts were prepared, and run on an SDS-Page gel. The resulting gel, stained with Coomassie blue, is shown opposite. From sequence data the predicted size of the fusion protein is 82 kd, however as XrelA protein runs anomalously high for its size the fusion product may run somewhat higher on the gel than predicted.

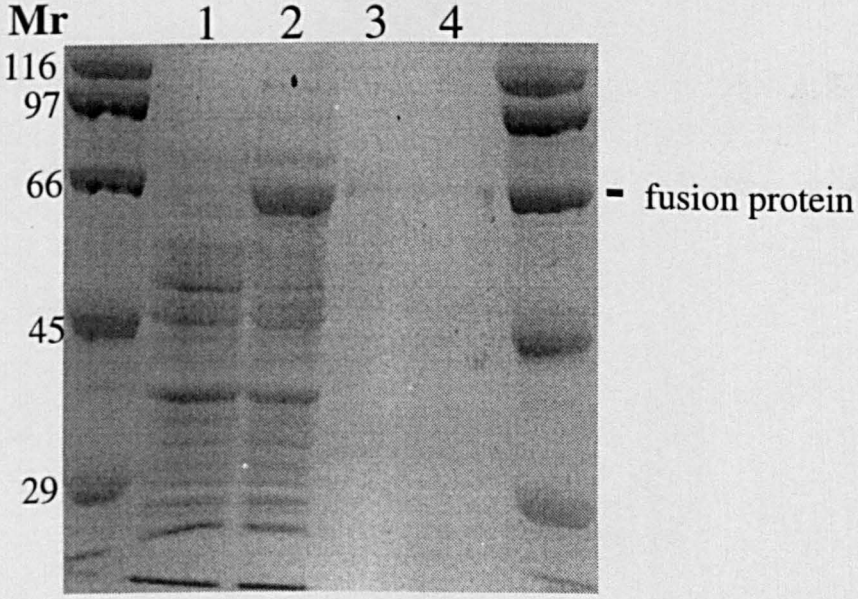
FIG. 14:- Purification of pET3a-XrelA fusion protein for use as an antigen.

SDS PAGE gel showing induction of pET-3a526 fusion protein expression with IPTG (1 and 2), and purification of fusion protein by electroelution (eluate shown in lane 3), and the final preparation after treatment to remove SDS.

pGEX-2TXR does not express detectable levels of fusion protein.



Purification of pET-3a526 fusion protein for use as antigen.



Extracts:-

1. Uninduced culture; 2. Induced culture;
3. After electroelution; 4. Final preparation.

The serum was then tested by Western blotting, as described in section 2.3.8., using extracts from oocytes injected with XrelA mRNA. After 10 boosts the serum from one rabbit was capable of detecting a band of the same size as XrelA in Western blots of extracts from uninjected oocytes (see figure 15). This rabbit was then terminated by terminal bleeding from a cardiac puncture in order to collect the maximum volume of serum. From this point serum is refered to as B175.

3.2.3. Testing the antiserum by immunohistology.

The ability of B175 to detect XrelA protein in embryonic tissue was tested by whole mount staining, as decribed in section 2.3.9., to embryos injected with XrelA mRNA and to uninjected embryos from a range of stages. Overexpressed XrelA protein was easily detected by these methods and was shown to be predominantly localised to nuclei (see figure 16). Unfortunately, no localisation of endogenous XrelA could be detected. As staining with pre-immune serum gave a similar level of background it seems likely that high background due to the a non-rel specific binding activity present in B175 which originated in the pre-immune serum (see figure 15), was responsible for obscuring any signal from localised of XrelA protein.

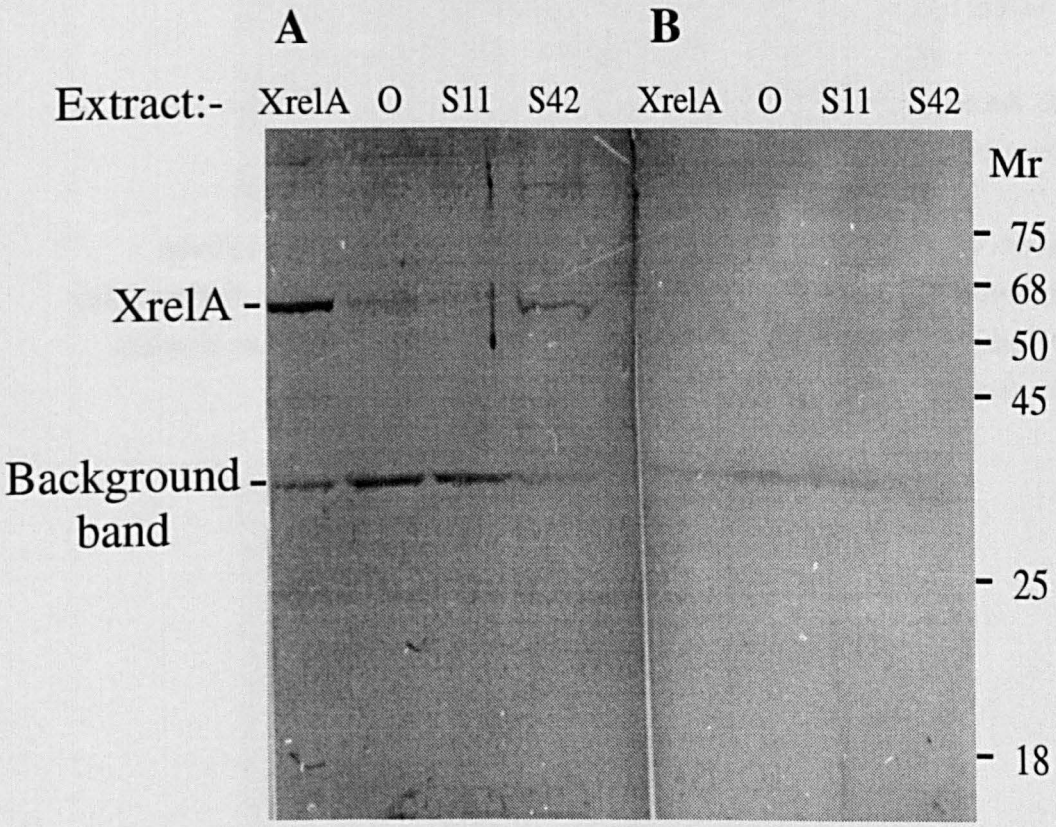
FIG. 15:- Western blots showing detection of exogenous and putative endogenous XrelA protein in oocytes and embryos.

Protein extracts made as described in section 2.3.1. were run on a mini SDS-PAGE gel at approximately 1/3 oocyte equivalent per lane. Western blots were carried out as described in section 2.3.8. using a 1/500 dilution of antiserum/pre-immune serum, and a 1/2000 dilution of HRP-Goat anti-rabbit as a secondary antiserum.

15A and **15B** show identical filters probed with **(B)** pre-immune serum and **(A)** B175 (final bleed antiserum).

Note the presence of the lower background band, which is also present in the pre-immune serum. XrelA itself runs at about 66 kd. Faint bands of this size can be seen in oocyte and stage 11 lanes, with a stronger band of that size in the stage 42 lane.

Western Blot showing putative detection of endogenous XrelA protein.



Extracts:-

XrelA :- Extract from oocytes expressing XrelA.
O :- Uninjected oocyte extract.
S11 :- Extract from stage 11 embryos.
S42 :- Extract from stage 42 embryos.

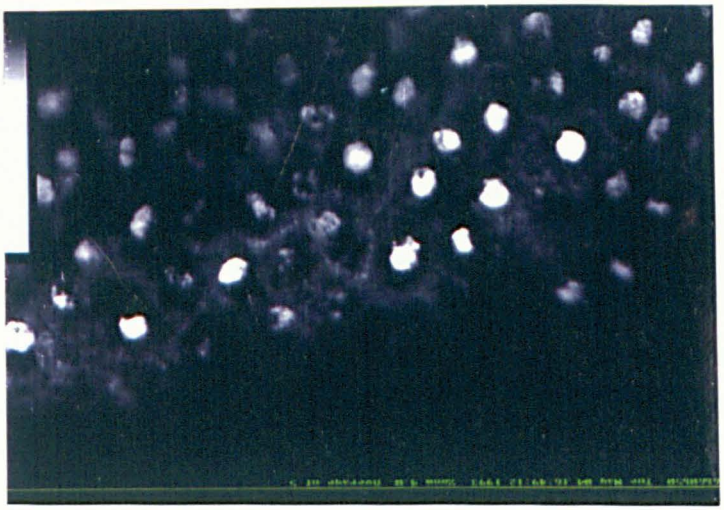
FIG. 16:- B175 shows nuclear localisation of exogenous XrelA in embryos, but no endogenous XrelA can be detected over background levels.

A. An optical section (by confocal microscopy) through the animal cap of a stage 9 embryo which was injected with 500 pg of XrelA mRNA at the two cell stage, after whole mount incubation with B175 (anti-XrelA) (1/150) and an FITC-conjugated goat anti rabbit IgG secondary antibody (1/200). Nuclear localisation of the exogenous protein is clearly visible.

B. An optical section through the animal cap of a control embryo, stained as described in **(A.)**.

Note:- No differential nuclear staining (fluorescence) is visible in these embryos. In fact the level of staining is indistinguishable from that seen after staining of control embryos in which B175 was substituted for an identical concentration of pre-immune serum (data not shown).

A



B

3.3. Conclusions and further work.

As discussed above, the inability of B175 to detect localisation of endogenous XrelA protein by immunohistology is probably due to the presence of a background, non-rel specific activity, which is also present in the pre-immune serum. This problem might feasably be overcome by affinity purifying the antiserum, which would, of course, require a pure source of protein. Given the problems encountered with expression of stable GST-XrelA fusion protein, and the fact that by this point the localisation pattern had already been characterised, I decided to concentrate my efforts elsewhere. Future attempts to produce pure protein for this or other purposes could make use of shorter affinity purifiable fusions, such as those produced by pQIA vectors (Diagen)[17].

[17] These vectors allow the production of fusions with a tag of six histidine residues which can be purified by Nickel chelate chromatography. Like the pET fusion vectors expression is induced via T7 RNA polymerase so preventing expression prior to induction.

4. Detecting endogenous κB binding activity: Gel Mobility Shift Assays.

4.1. Introduction

The development of an assay for active XrelA and its subsequent use to establish the spatial and temporal distribution of active protein in the embryo is of obvious significance for establishing the role of XrelA in embryogenesis. It is also crucial as a tool for testing the effectiveness of methods for inhibiting the activity of both exogenous and endogenous XrelA, and of any other rel family members which may be present in the embryo (see chapter 5.).

Gel mobility shift assays (GMSA's) are widely used to detect specific DNA binding activities in cell and embryo extracts. In these assays a radioactively labelled double stranded DNA probe is incubated with a protein extract, and run on a non-denaturing polyacrylamide gel. If the probe binds to proteins or multi-protein complexes present in the extract, its electrophoretic mobility is shifted. Specificity can be confirmed by the use of competition assays using an excess either of unlabelled probe, to compete out specific binding, or of a mutant competitor in which specificity defining residues have been altered, in order to compete out binding of undesired specificity. It is also possible to test for the presence of particular proteins in specifically binding complexes by using antibodies to further shift electrophoretic mobility, a phenomenon known as supershifting. As the conditions required for DNA binding have to be found empirically, the detection of individual binding activities often requires significant optimisation.

As discussed in the introduction (section 1.1.1) the paradigm for the activation of rel family members involves the signal dependent release of a rel dimer, which is held in the cytoplasm by an inhibitory protein of the IκB family. Binding of this inhibitor protein not only prevents nuclear localisation of the rel dimer, it also inhibits binding to DNA. Assuming that this is the case for the endogenous XrelA containing complex(es), it should be possible to use GMSA's to map the temporal and spatial distribution of activated XrelA containing complexes during development. XrelA containing complexes should be distinguishable from other κB binding complexes by supershifting with B175 antiserum. It is important to note that the rel family is not the only group of transcription factors capable of binding specifically to κB sites

(discussed on page 98), so any complexes which do not supershift do not necessarily represent other rel family dimers.

A previous, unsuccessful attempt to detect endogenous κB binding activity attributable to an XrelA containing complex in *Xenopus* embryos (Richardson, 1991) had used three different probes. One of these probes was based on a Dorsal binding site from the *zen* promoter, another on a κB site in the Interleukin-2 receptor promoter, and a third on an idealised pallindromic site (see figure 17). It is now known that not all rel dimers will bind to these sites (see introduction: page 10 and figure 4). For this reason it was decided to repeat these experiments with a different probe using a variety of conditions in an attempt to increase sensitivity, and also to use a mutant competitor to confirm specificity. In case the period during which the endogenous complex is active during development is brief, it was also decided to test a comprehensive stage series of extracts.

The choice of probe for a GMSA to detect active endogenous XrelA is complicated by the fact that the form which XrelA containing dimers might take in the embryo is unknown. As discussed in the introduction (section 1.1.3.), rel heterodimers have been found to bind to a wide range of promoter elements (see figure 4). The use of PCR selection assays has further increased the number of sequences known to bind to rel family dimers, as well as finding some sequences that are specific for particular rel dimers. As the κB site found in both the HIV-LTR and the κ B light chain promoter binds to the widest range of vertebrate rel complexes so far tested this site was an obvious choice for attempting to repeat this work. Another reason for using this site comes from the fact that it was used as a probe in the only study in which an NF-κB-like activity has been detected in *Xenopus*, although this activity was detected in oocytes rather than in embryos (Dominguez *et al.*, 1992). During this study of the mechanisms of oocyte maturation, it was found that nuclear extracts from oocytes treated with insulin to induce maturation contained a specific κB binding activity. This activity was found to be inhibitable by anti-p50 antisera and by IκBα which is known to require the presence of a c-rel, relB, or p65 subunit in order to bind to a rel dimer (see introduction section 1.1.1.). An apparently identical activity was also detected in deoxycholate (DOC) treated cytoplasmic extracts from untreated oocytes. As DOC is capable of dissociating IκBα from NF-κB (Dominguez *et al.*, 1992), this suggests that the rel complex detected is retained in the cytoplasm of unstimulated oocytes by interaction with an IκB like molecule.

FIG. 17:- Probes used in GMSA's:-

(A) As used in Richardson *et al.*, 1991

PALLINDROMIC :- CAACGGCAG***GGGAATTCCC***CTCTCCTT

IL-2R κb Site:- CAACGGCAG***GGGAATCTCC***CTCTCCTT

Dorsal (ZEN):- GTTTT***GGGAAATCCA***GAAG

(B) As used in this study

SINGLE SITE WT :-
(SKBW) CAACGGCAG***GGGACTTTCC***CTCTCCTT

SINGLE SITE MUT :-
(SKBM) CAACGGCAG***CTCACTTTCC***CTCTCCTT
***

DOUBLE SITE WT:-
(NFKBW) ACAA***GGGACTTTCC***GCTG***GGGACTTTCC***AGGA

DOUBLE SITE MUT:-
(NFKBM) ACAA***CTCACTTTCC***GCTG***CTCACTTTCC***AGGA
*** ***

WT = Wild Type; MUT = Mutant; All binding sites and mutated binding sites are shown in italics.

The complete sequence of the wild type double site probe is identical to a region of the HIV-LTR. In the other probes, however, sequences flanking the binding sites were chosen randomly. Note that, of the residues substituted in the mutant competitors (marked with a '*'), guanosine residues at positions 1 and 3 have been shown to be essential for p50 homodimer binding (Kunsch *et al.*, 1992).

It was important to try to replicate these experiments for a number of reasons. Firstly, as a preliminary to assaying for the same, or similar activity present in embryos, but also to test whether the complex in question contained XrelA, which should be easily tested by supershifting with B175.

4.2. Results

Protein extracts were made from oocytes or embryos as described in section 2.3.1. and stored at -70°C. Gel Mobility Shift Assays were carried out using the same method used by the Dominguez group (Laura Sanz, personal communication) as described in section 2.4., using oligonucleotide probes labelled as described in section 2.4.2. Unless otherwise stated, the equivalent of one oocyte of protein extract (approximately 20 μg of soluble protein) was used per incubation. Optimisation experiments were carried out where necessary, varying the NaCl concentration in the binding buffer, and the p(dI-dC) (non-specific competitor) concentration. Unless otherwise stated, all assays were carried out in the presence of 100-fold excess of mutant competitor.

4.2.1. A constitutively active, nuclear localised specific κB binding activity can be detected in oocytes.

Oocytes were treated with 1μM insulin (in 0.1 % BSA in BX) at 19°C and after about 4 hours half the oocytes were harvested. The remaining oocytes were left overnight in insulin and then scored for germinal vesicle breakdown (a marker of maturation visible as a white spot in the animal hemisphere). Nuclei were isolated from the harvested oocytes by manual dissection, and their extracts concentrated using Centricon 30 tubes (see section 2.3.1.), and the concentration of soluble protein determined as in section 2.3.3. Extracts from the enucleated oocytes were prepared as for whole oocytes. GMSA's were carried out, using the NFKBW probe (as used by Dominguez and colleagues), to compare the binding activities present in treated and untreated nuclear extracts (10 μg/binding reaction) and to test for DOC unmaskable binding activities in the cytoplasm of untreated oocytes. It is important to note that the use of a double κB site probe complicates the data, as two bands, representing single and double site occupancy, are seen for each binding activity.

As can be seen from figures 18 and 19, no new binding activities were detected in nuclear extracts from insulin treated oocytes compared to controls. This was despite the fact that the concentrations of insulin used were able to induce maturation of 24/28 oocytes after 24 hours compared to 1/30 controls. Titration of the concentration of the non-specific competitor, in the form of poly (dI-dC), failed to unmask any hidden activity. Similarly, no κB binding activity was unmasked in cytoplasmic extracts in the presence of DOC. However, a doublet of bands was

consistently seen in nuclear extracts and in whole oocyte extracts. This doublet could be competed out with wild-type competitor, leaving a single band, much fainter than either of the original two. The doublet could represent one or two specifically binding complexes.

In conclusion, oocytes were found to contain one (or possibly two) constitutively active κB specific DNA binding complexes, which are localised to the nucleus. Unlike Dominguez and colleagues I found no evidence for the existence of a further κB binding complex in oocytes activated by insulin treatment.

4.2.2. B175 is capable of supershifting exogenous XrelA homodimers, but not endogenous κB binding complexes found in oocytes.

In order to test whether the antiserum B175 could supershift the endogenous binding activities found in oocytes, GMSA's were carried out using oocyte extracts which were pre-incubated with or without B175, prior to the addition of oligonucleotide probe (NFKBW). As a positive control, the same procedure was carried out with extracts from oocytes injected with XrelA RNA, and as a control for binding activities which might be present in the antiserum, a further assay was carried using antiserum but without any extract. The results of this experiment can be seen in figure 20. A doublet corresponding to the endogenous binding activities can be seen in all lanes apart from the negative control. No new bands result from the addition of antiserum to uninjected oocyte extract. Two bands can be seen in the XrelA injection lane, representing single and double site bound probe. In the presence of antiserum these bands are reduced in intensity, and two new slow migrating bands can be seen, presumably representing supershifted double and single site bound probe These bands are not seen in the control lanes.

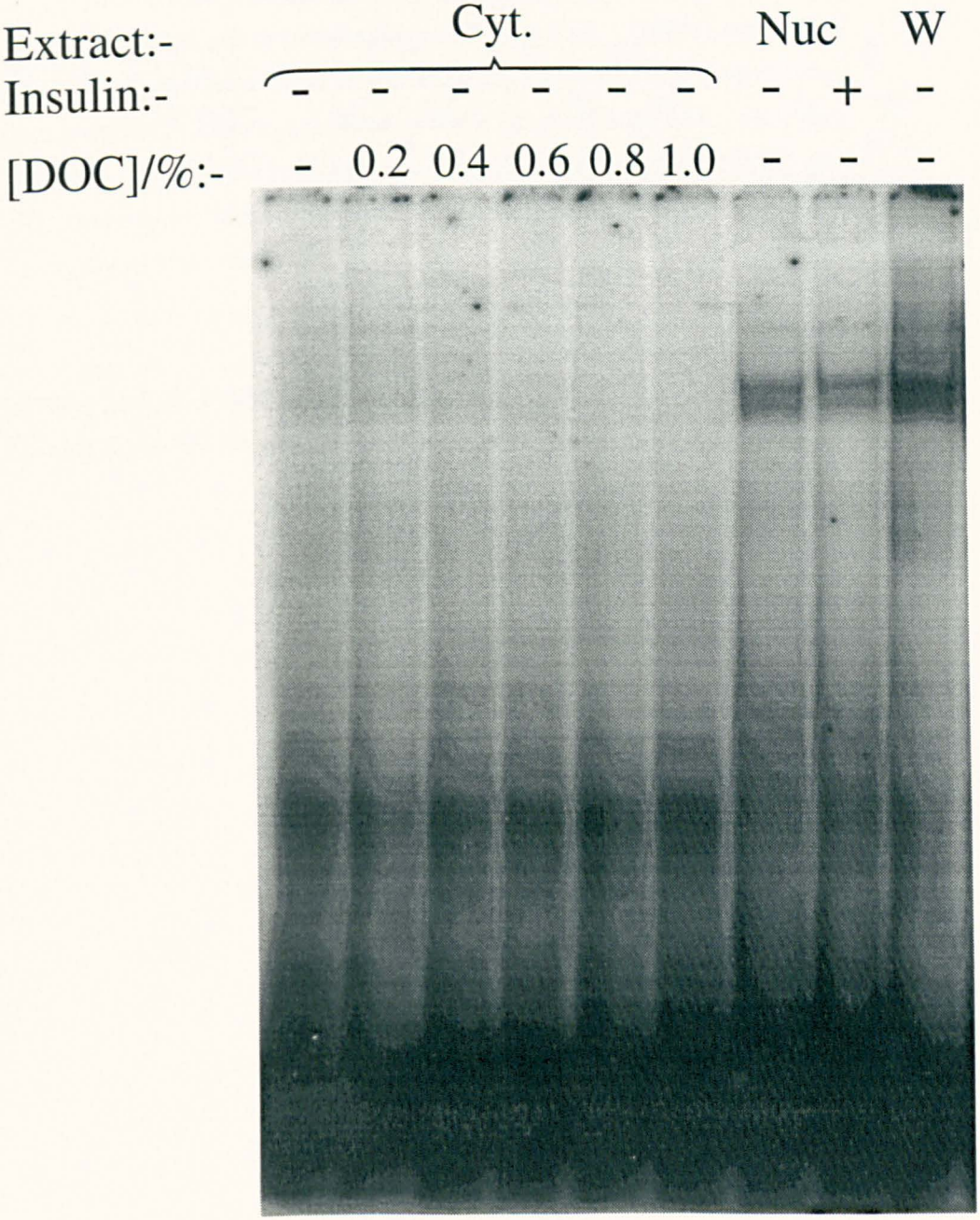
The supershifting of exogenous XrelA homodimers bound to probe shows that B175 antiserum can be successfully used to identify XrelA containing κB binding complexes, under the conditions used. As the endogenous binding complex is not supershifted it seems safe to assume that this complex does not contain endogenous XrelA protein.

FIG. 18:- GMSA to detect insulin activated κB binding complex.

To show that the concentration of insulin used was capable of inducing oocyte maturation, oocytes were treated with 1 μM insulin (SIGMA) for 24 hours at 19°C and then scored for germinal vesicle breakdown (GVBD) which is visible as a white spot in the animal hemisphere. From the treated group 24/28 had clearly undergone GVBD, compared to 1/30 untreated oocytes. Cytoplasmic and nuclear extracts were made from oocytes incubated +/- insulin for 4 hours. These extracts were then used in GMSA with NFKBW probe to assay for any activation of κB binding activity in nuclei by insulin, and for the presence of κ B binding activities in the cytoplasmic extracts unmaskable by sodium deoxycholate (DOC) treatment.

No binding activities can be seen in untreated extracts, and none were unmasked by DOC treatment at any of the concentrations used. No new bands can be seen in the insulin treated oocyte nuclear extract lane compared to the untreated oocyte nuclear extract lane, although a doublet which be seen in the whole oocyte lane is also seen in both of these.

GMSA to detect insulin activated kB binding complex



EXTRACTS:-

Cyt. = Cytoplasmic extract
Nuc.= Nuclear extract
W=Whole Oocyte extract

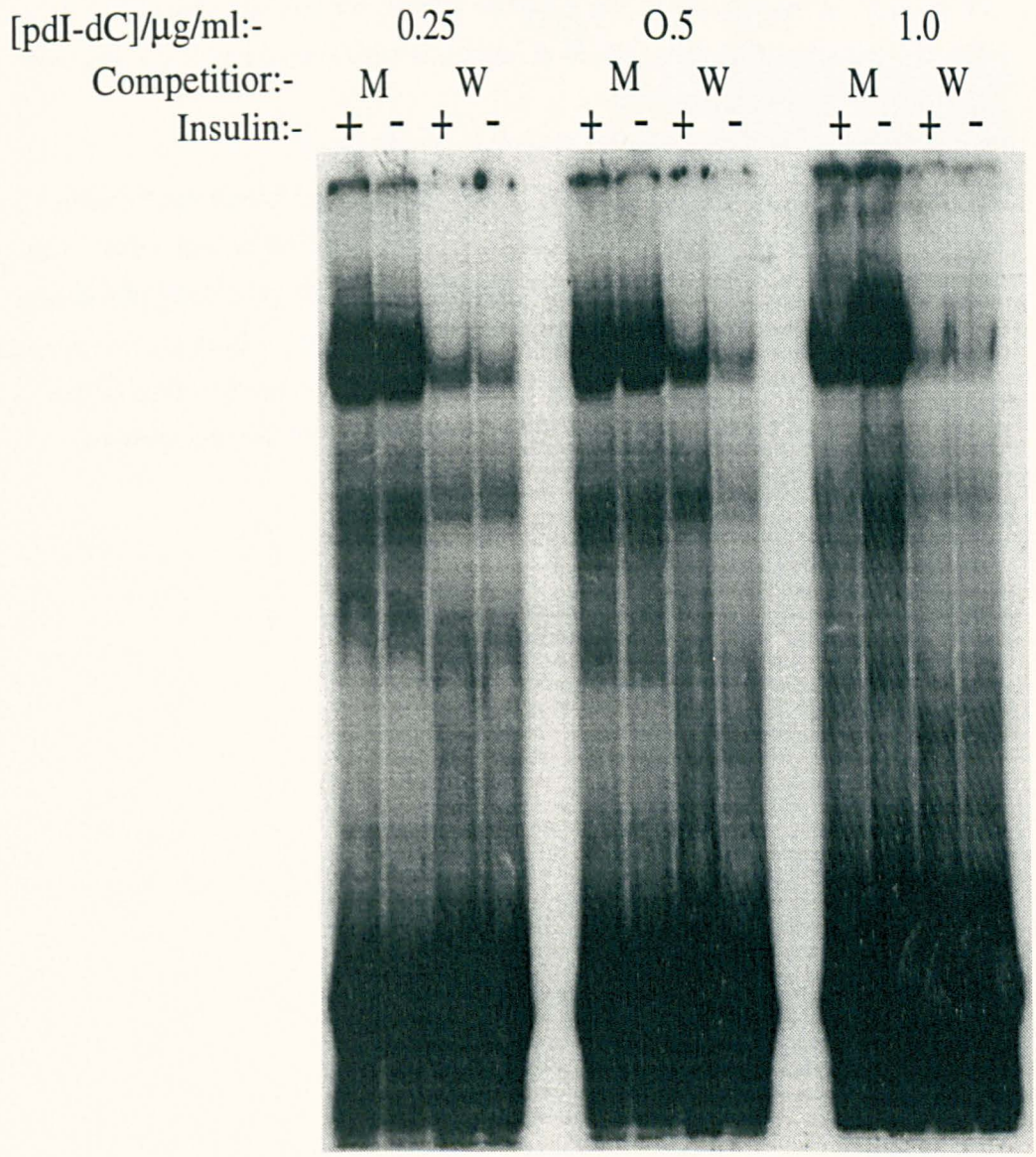
DOC = sodium deoxycholate

FIG. 19:- Oocytes contain a constitutive κB binding activity.

Oocytes were treated for 24 hours at 19°C with 1 μM insulin in BX containing 0.1% BSA (Fraction V, Sigma). After this time 22/25 treated oocytes showed clearly visible evidence of germinal vesicle breakdown (GVBD), whereas out of 25 untreated controls none underwent GVBD. Extracts of oocytes treated in the same way for 8 hours were made to test for the presence of insulin-activated κB binding activities. The specificity of binding activities present in these extracts was confirmed by adding a 200-fold excess of cold probe to half the binding reactions. The concentration of non-specific competitor (pdI-dC) was also titrated in the hope that this might reveal any binding activities masked by non-specific binding.

This experiment confirms that the constitutively active binding activity is κB specific, but as in the previous experiment, no insulin-activated κB binding complexes can be seen.

Oocytes contain a constitutively active κB binding activity.



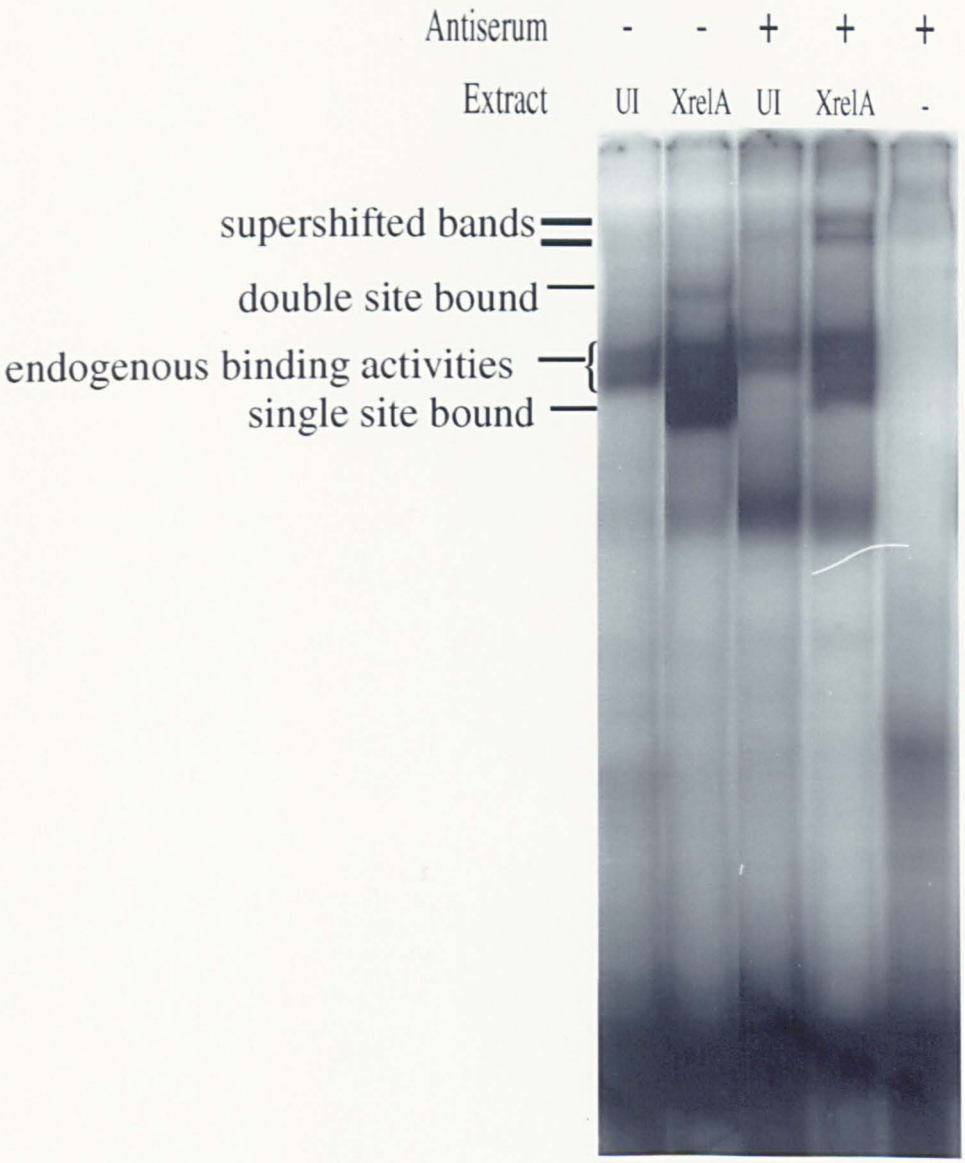
Probe :- Double κB site (NFKBW)
Competitors:- M=Mutant, W=Wild-type
both used at 100 fold excess over probe.

FIG. 20:- B175 is capable of supershifting exogenous XrelA homodimers, but not endogenous κB binding complex found in oocytes.

Extracts were made from uninjected oocytes, or oocytes injected with RNA encoding XrelA and incubated overnight. These were then used in GMSA's with double site probe (NFKBW; see figure 17) +/- preincubation with B175 antiserum (1 μl/incubation). As a further control for binding activities originating in the antiserum, binding reactions with antiserum in the absence of extract were also carried out.

Because a double site probe was used in this experiment bands can be seen representing XrelA homodimers bound to a single site and to both sites. The addition of B175 antiserum leads to a decrease in intensity of these two bands and the appearance of two new, slower migrating bands, which are not present in the control lane. These bands are likely to be due to supershifting of the single and double site bound probe. No supershifting of the endogenous binding activities can be seen.

Supershifting of XrelA by b175 antiserum.



Extracts:-
XrelA - Extract from oocytes injeced with 2 ng XrelA mRNA
UI- Extra ct from uninjected oocytes
Probe:- Double kB site (NFKBW)

4.2.3. Three specific κB binding activities are present in embryos.

To study the temporal distribution of κB binding activities in embryos, protein was extracted from a comprehensive stage series of embryos, and assayed for soluble protein. These extracts were then used in GMSA's using SKBW probe and an extract concentration of 20 μg of soluble protein per binding reaction. The results of this experiment are shown in figures 21 and 22. Of the seven distinct binding activities detected only three are completely competed out by the addition of a 100-fold excess of wild type competitor (cold probe), indicating that they represent binding activities specific for the HIV-LTR type κB site. These three binding activities are referred to from here onwards as κB1, κB2 and κB3 (see figure 21). A fourth binding activity (the top band in each lane), was reduced but not eliminated by wild-type competition. None of these, however, has a temporal pattern of activation that corresponds to what would be predicted from the data on nuclear localisation of XrelA protein, i.e. activation during blastula stages followed by loss of activity around stage 10 (Bearer, 1994). The levels of activity of κB2 and κB3 appear to vary with stage. κB2 activity increases steadily up to the mid/late neurula before levelling off. κB3 activity, although present in unfertilised eggs, appears to increase slightly in level in the late blastula and then tails off in the neurula stages. This latter result has, however, proved difficult to repeat, as there is often significant variation between experiments, in the levels detected compared to other bands, presumably because of a lack of stability of the complex. Consequently the variation in binding with stage seen here may be artifactual.

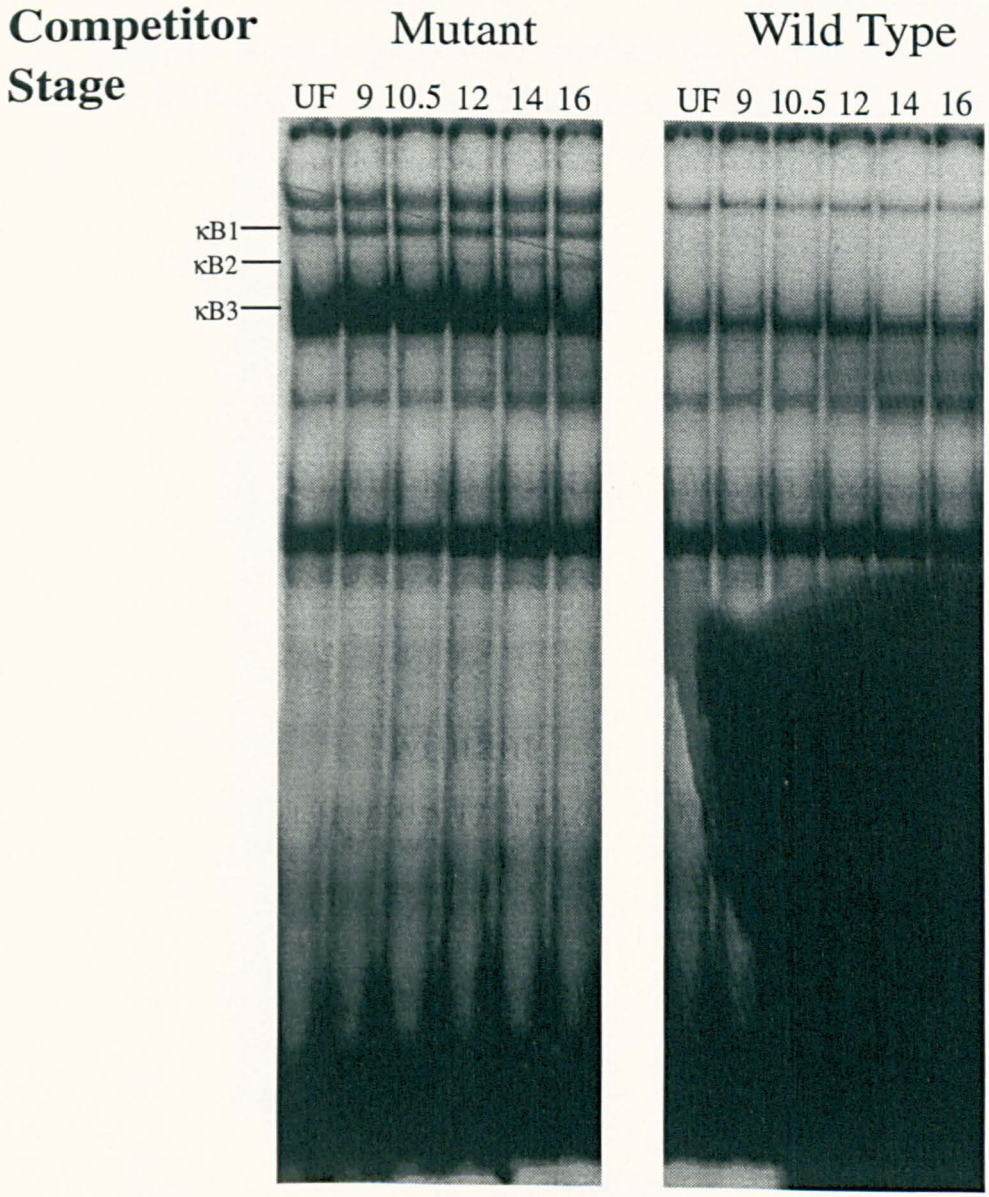
In order to test for the presence of any κB binding complexes whose activity might be masked by binding to an IκB family member, extracts from all stages were pre-incubated with and without DOC (0.8 mM) before the addition of probe. No new binding activities were uncovered, and none of the existing activities was noticeably enhanced by this treatment (see figure 23). One of the main aims of this work was of course to try to detect an endogenous κB binding complex containing XrelA protein. To test whether any of the κB binding complexes detected contained XrelA protein, extracts were pre-incubated with and without the antiserum B175 (1 μl per extract) prior to the addition of probe. As can be seen in figure no supershifting of any of these bands was observed.

FIG. 21:- GMSA showing κB binding complexes present in embryos from fertilisation to stage 16.

This figure shows GMSA carried out using a protein extracts from various stages (as indicated) from fertilisation to stage 16. Extracts were assayed for soluble protein, and binding reactions were carried out using 20 μg of soluble protein per reaction.

Note there are (at least) three bands which are competed out by the addition of wild-type competitor, but not by mutant competitor, indicating that these bands represent κB-specific binding complexes. These are referred to as κB1, κB2 and κB3 with κB1 being the slowest migrating and κB2 the fastest Of these activities κB2 and κB3 vary with stage. κB2 is first seen in the early gastrula, after which levels gradually rise up to the mid-neurula. Active κB3 is present at all stages, but levels seem to decrease somewhat after gastrulation.

GMSA showing κB binding complexes present in embryos.

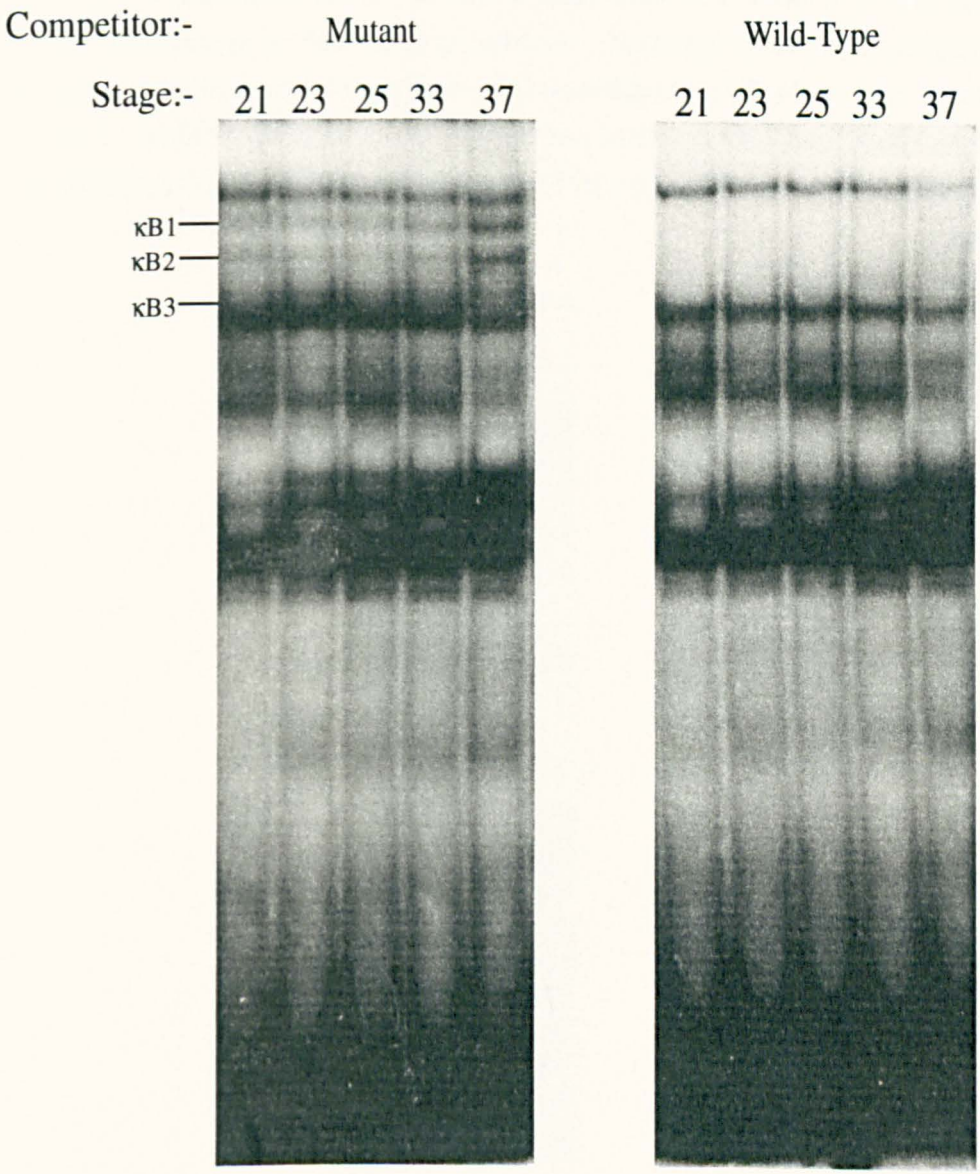


UF = Unfertilised
Probe :- SKBW
κB1, 2 & 3 are specific binding activities

FIG. 22:- GMSA showing κB binding activities present in embryos between stages 21 and 37.

This figure shows GMSA carried out using a protein extracts from various stages (as indicated) from stage 21 to stage 37. Extracts were assayed for soluble protein, and binding reactions were carried out using 20 μg of soluble protein per reaction.
The same three κB specific binding complexes can be seen in these extracts as in extracts from earlier stages (see figure 21). No differences in binding levels of these complexes can be seen over these stages.

Stage series showing specific κB binding activities present between stages 21 and 37



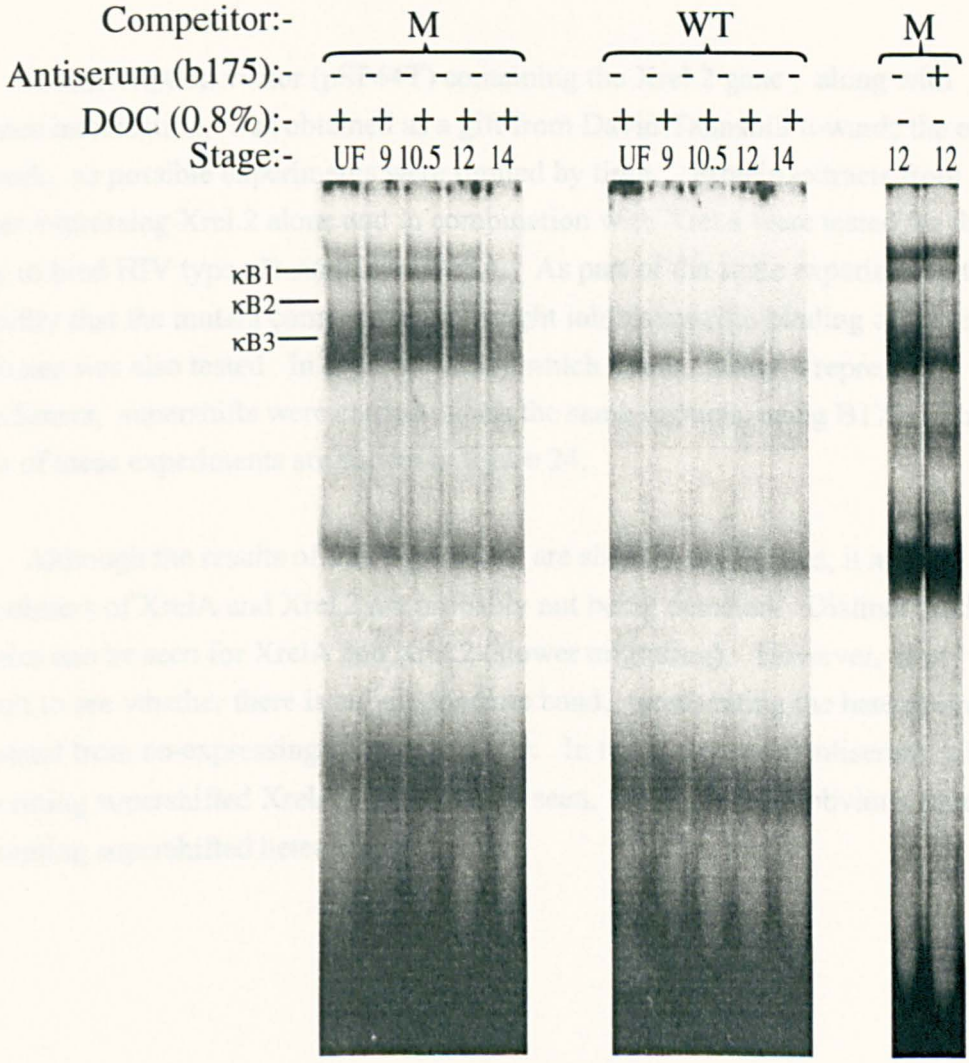
Probe:- SKBW

κB1, 2 & 3 are specific binding activities

FIG. 23:- GMSA showing that no binding activities are unmasked in embryo extracts by DOC and that endogenous κB binding activities do not supershift with B175.

Extracts from stages as indicated were pre-incubated prior to addition of the probe with or without DOC (0.8 %) and with or without antiserum B175 (1 μl/binding reaction) as indicated. No binding activities are apparent in the extracts treated with DOC in addition to those already characterised. None of the specific κB binding activities previously characterised (see figure 21 for comparison) are supershifted by B175 antiserum indicating that none involves XrelA.

GMSA showing that no binding activities are unmasked in embryo extracts by DOC and that endogenous κB binding activities do not supershift with B175.



Probe:- SKBW
UF=Unfertilised
DOC = Deoxycholate
M = Mutant competitor
WT = Wild-Type competitor

κB1, 2 & 3 are specific binding activities

4.2.4. Interaction of Xrel.2 with XrelA.

The only likely heterodimerisation partner for XrelA known to be present in the embryo is a novel *Xenopus* member of the rel family known as Xrel.2 (see introduction, page 19). As discussed later (section 4.3.) one possible explanation for the lack of detection of endogenous XrelA using GMSA's is that XrelA is present as a dimer with Xrel.2 and that this heterodimer is unable to bind to HIV-LTR type κB sites.

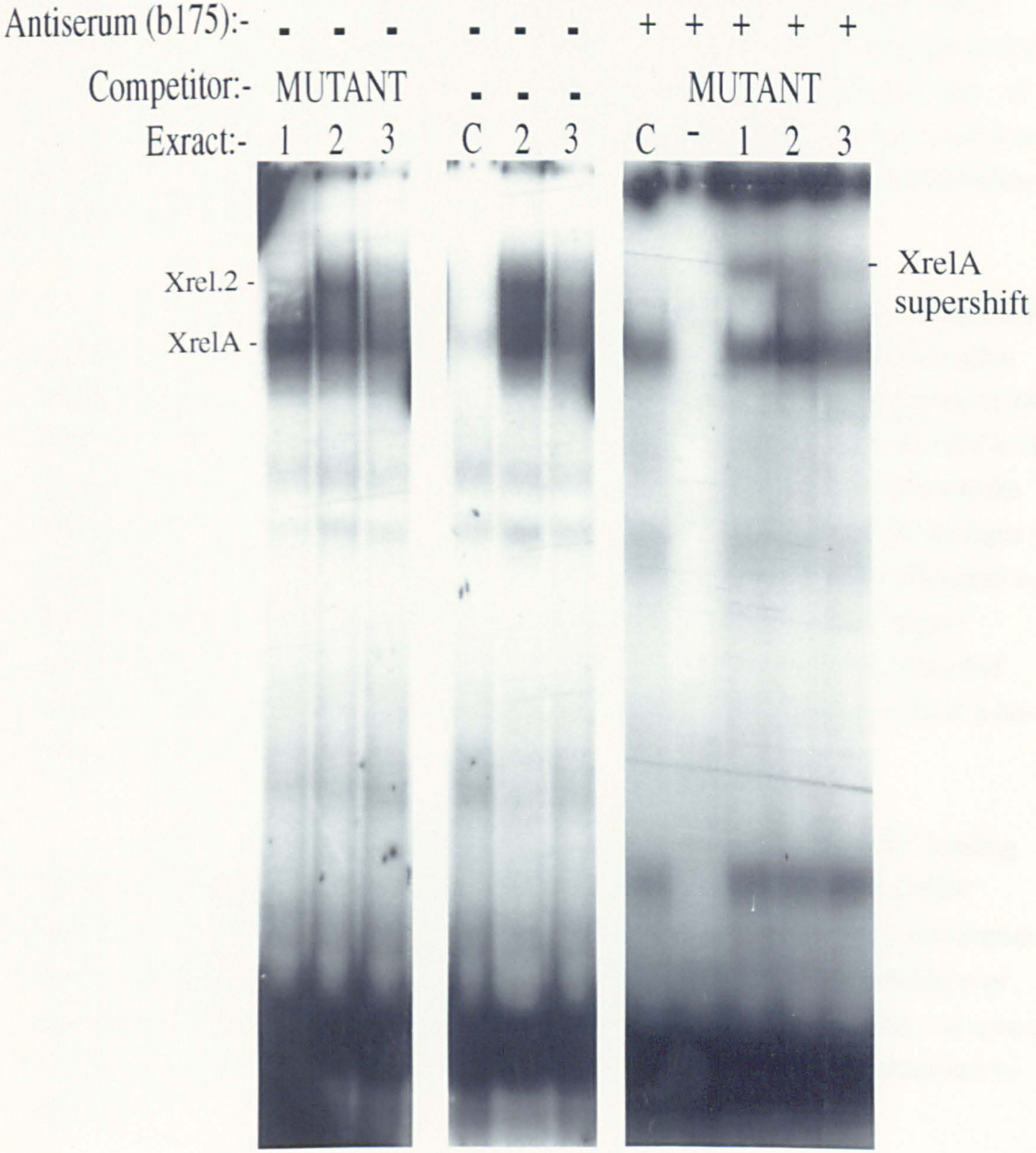
A transcription vector (pSP64T) containing the Xrel.2 gene , along with sequence information, was obtained as a gift from David Tannahill towards the end of this work, so possible experiments were limited by time. Protein extracts from oocytes expressing Xrel.2 alone and in combination with XrelA were tested for their ability to bind HIV type κB sites in a GMSA. As part of the same experiment, the possibility that the mutant competitor used might inhibit specific binding of these complexes was also tested In order to clarify which bands, if any, represent heterodimers, supershifts were carried out on the same extracts, using B175. The results of these experiments are shown in figure 24.

Although the results of this experiment are slightly ambiguous, it appears that heterodimers of XrelA and Xrel.2 are probably not being detected. Distinct binding activities can be seen for XrelA and Xrel.2 (slower migrating). However, it is difficult to see whether there is an intermediate band, representing the heterodimer, in the extract from co-expressing oocytes (lane 3). In the presence of antiserum, a band representing supershifted XrelA can be clearly seen, but there is no obvious band representing supershifted heterodimer.

FIG. 24:- Xrel.2 / XrelA heterodimerisation and supershifts.

Extracts were made from oocytes injected with XrelA or Xrel.2, or a combination of the two and assayed for their ability to bind to the probe SKBW by GMSA, and for supershifting by B175.
Distinct binding activities can be seen for XrelA and Xrel.2 (slower migrating), but smearing means that it is not clear whether a heterodimer band is present. In order to clarify the situation supershifting of these binding activities was investigated. The fact that no band representing supershifted heterodimer can be seen suggests that it may have a different binding specificity.

GMSA showing DNA binding and heterodimerisation of Xrel.2



1 1ng XrelA
2 1ng Xrel.2
3 1ng XrelA + 1ng Xrel.2

4.3. Conclusions, discussion and future work.

4.3.1. Possible reasons for failure to detect an endogenous XrelA containing complex.

A number of explanations could be proposed for the failure to detect an endogenous relA containing κB binding activity. Exogenously expressed XrelA homodimers tend to appear as a smudge rather than a distinct band on the gel making detection even of low levels of exogenous homodimer difficult (see figure 27). If most of the XrelA protein is present as homodimers it is possible that the assay used is not sensitive enough. It is also possible that XrelA is present in a novel heterodimer, with novel DNA binding specificity.

The only rel family member known to be present in the embryos besides *XrelA* is *Xrel.2*. Overall *Xrel.2* has most similarity to c-*rel* (65% inclusing conservative substitutions), although this similarity is significantly less than the similarity of *XrelA* and *relA*. The one relA containing heterodimer which does not bind to an HIV-LTR type κB site is relA/Rel, which binds instead to a urokinase promoter type κB site (Hansen *et al.*, 1992). Probes with this site may be a good bet for future attempts to detect an active endogenous XrelA containing complex. However, as discussed in the introduction (see page 19), *Xrel.2* has some novel substituions in a highly conserved region of the rel homology domain (RHD) implicated in the control of DNA-binding specificity, which opens up the possibilty that Xrel2. may have a novel range of specificity.

Finally, another possible explanation for the lack of detection of a binding complex is suggested by work on the effects of NF-κB interaction with C/EBP proteins (see page 151). NF-κB interacts physically with a number of transcription factors belonging to the C/EBP family. This interaction leads to a potentiation of binding to C/EBP sites, but an inhibition of binding to HIV type κB sites, as tested by GMSA (Stein *et al.*, 1993). No C/EBP proteins have so far been characterised in *Xenopus*.

4.3.2. Speculations on the identity of specific κB binding activities detected in embryos.

As mentioned in the introduction to this section, GMSA using κB site probes are likely to detect both rel and non-rel specific κB binding complexes. Non rel family

κB specific binding activities include EBP1, HIVEN86A, zinc finger protein H2TF1 (possibly ubiquitous), and the two related zinc finger proteins αA-CRYBP1 and AGIE- BP1 (Faisst and Meyer, 1992). The work described in the following chapter using rel specific dominant negatives suggests that two of the activities detected (at least) may involve such proteins (see section 5.5)

5. Inhibiting XrelA: The design, construction and testing of dominant negative interference clones.

5.1. Introduction: strategies for specific elimination of gene activity in *Xenopus*

One of the greatest drawbacks of using *Xenopus laevis* as a model system for studying early vertebrate development is the fact that the generation time is too long to make mutagenesis screening, or indeed any classical genetics, practical. In addition to this, the technology does not currently exist to allow targeted mutagenesis of *Xenopus* genes as it does for mice. This is especially frustrating when one considers the range of genes with developmentally significant activities isolated from *Xenopus* in functional screens.

Many attempts have been made to overcome this problem using methods which aim to specifically inhibit gene activity post-transcriptionally. One method which initially showed some promise was the use of antisense RNA, thought to block transcription specifically by hybridising specifically to its complementary transcript (reviewed in Weintraub, 1990). Unfortunately this approach has met with only limited success in *Xenopus*. Preliminary experiments with *Xenopus* oocytes showed that antisense RNA could be used to eliminate exogenous sense RNA (Harland and Weintraub, 1985), but equivalent experiments in embryos were not as successful. Initially this was thought to be due to the presence of a double stranded RNA unwinding activity in the newly fertilised *Xenopus* embryo, released from the oocyte nucleus during breakdown of the nuclear envelope (Bass and Weintraub, 1987). However, this 'unwinding' activity was later shown to be due to an enzyme now called double-stranded RNA deaminase, which converts about 25% of the adenosine residues in the target RNA duplex to inosines so destabilising the duplex and causing it to unwind (Bass and Weintraub, 1988; Polson and Bass, 1994). As such an activity acting on mRNA would turn message into nonsense, this activity cannot be responsible for the failure of antisense technology in *Xenopus* embryos, and the real reason remains a mystery.

Another approach to eliminating specific transcripts involves the use of antisense DNA oligonucleotides as primers to direct RNase H activity. As short DNA oligos are unstable *in vivo* due to endogenous exonuclease activities, oligonucleotides with modified internucleoside linkages (e.g.:- to phophoramidate) have to be used

(Dagle *et al.*, 1990). Such modified DNA oligonucleotides have been successfully used by Janet Heasman and colleagues to deplete pools of specific maternal RNA by injection into oocytes which are then subsequently labelled with dye (for identification purposes) and matured with progesterone before re-implanting into a primed host female (Heasman *et al.*, 1992 and1994). It should be noted, however, that this technique depends on the absence of significant stores of maternal protein.

By far the most popular and successful approach to the elimination of gene activity in *Xenopus* has been the use of dominant negative interference clones to inhibit the activity of gene products. This rather complicated sounding term (dominant negative for short) is adopted from classical genetics, where dominant alleles capable of inhibiting the activity of a wild-type allele of the same gene in a heterozygous organism are referred to as dominant negative alleles. The ideal targets for such an approach are gene products which are active as dimers, preferably with very specific dimerisation activity. Peptide growth factor receptors (PGF-R's) fulfil these requirements, and are also of obvious interest developmentally with respect to their role in transducing inductive signals. It is therefore not very surprising that the most interesting and fruitful work using dominant negatives in *Xenopus* has been their use in investigating the nature of inductive signals. PGF-R dominant negatives generally have truncations or deletions in the intracellular 'signalling' domain allowing them to act either by forming inactive heterodimers with endogenous wild-type protein or by sequestering the endogenous receptor's ligand. One of the advantages of PGF-R based dominant negatives is that they can be rescued by overexpression of the wild type receptor. This control confirms the specificity of the dominant negative, at least to the degree that its target(s) are functionally equivalent to the wild type receptor. Dominant negative interference clones have also been used to study the role of particular signal transduction pathway elements in mesoderm induction. The targets for the dominant negatives in this case, the GTP binding protein ras and the ser/thr kinase raf, are not dimeric proteins so a different approach is required. Instead the dominant negatives are designed so that they are incapable of further transducing the signal (e.g.:- in the case of raf, the kinase domain is inactive), but retain the ability to bind to upstream activators. When these clones are overexpressed they saturate the binding sites on these upstream activators, inhibiting signal transduction. This could potentially cause problems with interpretation as such dominant negatives could potentially be inhibiting upstream activator(s) with additional targets to their wild-type equivalents.

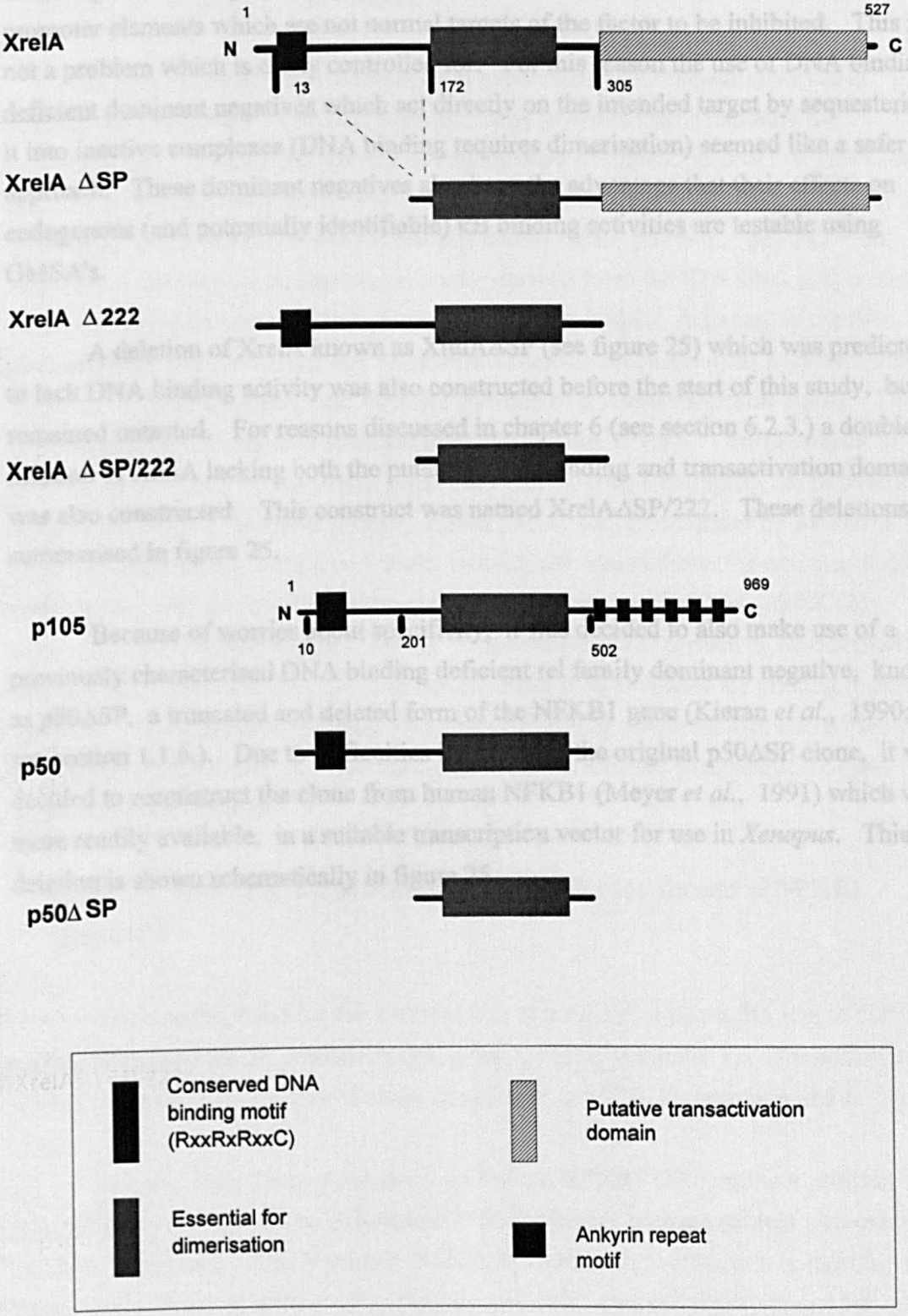
5.2. Design of rel family dominant negatives

XrelA protein can be detected in oocytes at levels approximately equivalent to those seen at blastula stages (Bearer, 1994). The relatively low levels of maternal XrelA message indicate that there is unlikely to be a rapid turnover of XrelA protein prior to MBT. If this is the case, then the pool of XrelA protein present in oocytes is likely to contribute significantly to the localised XrelA protein detected in the blastula. These considerations mean that the use of antisense modified DNA oligonucleotides to target XrelA protein in the blastula is probably not a viable approach.

There are a number of possibilities for designing dominant negatives based on XrelA. All rel family members, as far as is known, act as dimers, so any mutant XrelA capable of forming inactive dimers would be expected to act as a dominant negative. Unfortunately, members of the rel family are very promiscuous in their dimerisation abilities, so that, with the exception of relB, all known vertebrate members of the rel family are capable of heterodimerisation with each other (see introduction, section 1.1.1.). Consequently, any XrelA based dominant negative would be expected to target the activity of other rel family members if they are present, and not just XrelA and Xrel.2.

The two most obvious ways of producing a rel dominant negative are to specifically interfere with either DNA binding or transactivation without inhibiting dimerisation. Given the extent of conservation of the rel homology domain between relA and XrelA, and the extensive degree to which domains and residues necessary for the various activities of relA have been characterised (see section 1.1.6.), the design of deletion mutants lacking DNA binding or transactivation activity is relatively straightforward. XrelA homodimers have been shown to have the ability to activate transcription from an HIV-LTR based reporter (Richardson *et al.*, 1994). By analogy with relA one would expect the C-terminal region of the protein outside the rel homology domain to be necessary for this activity. A transactivation deficient mutant of XrelA, known as XrelAΔ222 (see figure 25), which lacks the C-terminal 222 amino acids was constructed, tested and characterised prior to the start of this work (Richardson, 1991; Richardson *et al.*, 1994). XrelAΔ222 is able to inhibit transactivation by exogenous XrelA from an HIV-LTR based reporter construct. This probably occurs by the saturation of promoter sites by homodimers of XrelAΔ222. It is also possible that XrelAΔ222 could act by forming transactivation deficient

FIG. 25:- Summary of deletions of XrelA and p105.



heterodimers with XrelA, although this is difficult to prove. Dominant negative transcription factors which act by saturating promoter sites have the disadvantage that, when expressed at highly non-physiological concentrations, they may interact with promoter elements which are not normal targets of the factor to be inhibited. This is not a problem which is easily controlled for. For this reason the use of DNA binding deficient dominant negatives which act directly on the intended target by sequestering it into inactive complexes (DNA binding requires dimerisation) seemed like a safer approach. These dominant negatives also have the advantage that their effects on endogenous (and potentially identifiable) κB binding activities are testable using GMSA's.

A deletion of XrelA known as XrelAΔSP (see figure 25) which was predicted to lack DNA binding activity was also constructed before the start of this study, but, remained untested. For reasons discussed in chapter 6 (see section 6.2.3.) a double deletion of XrelA lacking both the putative DNA binding and transactivation domains was also constructed. This construct was named XrelAΔSP/222. These deletions are summarised in figure 25.

Because of worries about specificity, it was decided to also make use of a previously characterised DNA binding deficient rel family dominant negative, known as p50ΔSP, a truncated and deleted form of the NFKB1 gene (Kieran *et al.*, 1990; see section 1.1.6.). Due to difficulties in obtaining the original p50ΔSP clone, it was decided to reconstruct the clone from human NFKB1 (Meyer *et al.*, 1991) which was more readily available, in a suitable transcription vector for use in *Xenopus*. This deletion is shown schematically in figure 25.

5.3. Construction of rel dominant negative clones.

5.3.1. Transcription vectors.

In order to carry out overexpression experiments in *Xenopus* embryos or oocytes, it is necessary to be able to consistently produce stable message for microinjection. To this end cloning strategies were designed, in order to insert just the coding region of each particular deletion/truncation (with a new stop codon if necessary) into a modified form of the expression vector pSP64T. pSP64T (see Appendix 1 for map) is an expression vector derived from the SP6 RNA polymerase based transcription vector pSP64 (Krieg and Melton, 1984). Adjacent to the SP6 promoter start site it contains the untranslated regions of the *Xenopus* β-globin gene separated by a cloning site, and with the addition of a polyadenylation signal. These untranslated regions serve to stabilise transcribed RNA *in vivo*. One drawback of pSP64T as a cloning vector is that it has only one cloning site (BglII), so to overcome this disadvantage a modified version, called pSPJC was constructed by Jonathon Cooke (unpublished), with a new multi-cloning site inserted into the existing single cloning site, and the deletion of the old multi-cloning site of pSP64 (pSPJC(2) construction is detailed in Appendix 1). Unfortunately this proved to be extremely limiting with respect to the choice of linearisation sites available for making message template. To improve this I inserted a new multiple linearisation site into a PstI site near to the terminal poly A. The resulting vector is referred to as pSPJC2L (See Appendix 1 for details).

5.3.2. Construction of a DNA binding deficient variant of NFKB1 (p50ΔSP).

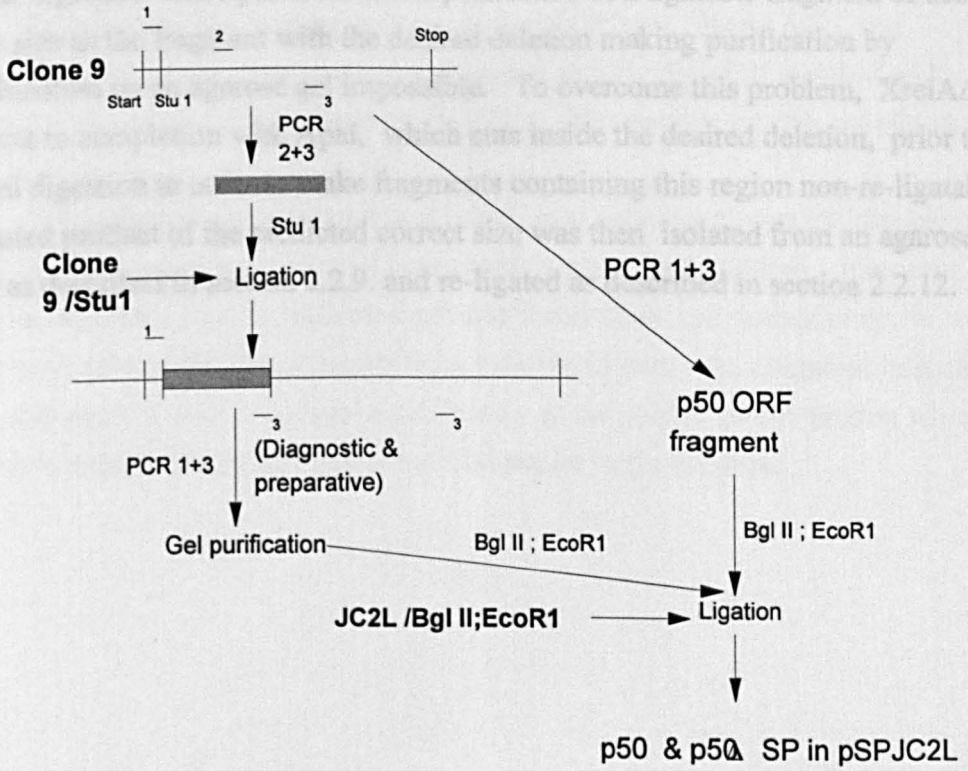
The starting point for the construction of p50ΔSP in was a full length cDNA clone of human NFKB1 in pBluescript called *C9* (see Appendix 1). The following strategy was used to construct a clone of p50ΔSP in pSPJC2L (summarised in figure26 (b)):-

Primers were designed to the 5' end of the NFKB1 ORF and to a position equivalent to the start of the C-terminal p105 inhibitory region which is cleaved off to produce active p50. The 3' primer (Primer 3) included a 5' extension consisting of a stop site followed by an StuI site, then by an EcoR1 site and finally by a 4 bp clamping sequence to facilitate cleavage at the adjacent site. The 5' primer (Primer 1) included a 5' extension containing a Bgl II site with a GC clamp. A further primer (Primer 2) was designed to the 3' end of the deletion with a 5'

FIG. 26:- (a)Primers for p50ΔSP construction and cloning into pSPJC2L vector.

PRIMER 1	Bgl II	GACTAGATCTATGGCAGAAGATGAT
PRIMER 2	Stu1	GACTAGGCCTCAGCAGACCAAGGA
PRIMER 3	Stu1 EcoR1 Stop	GACTAGGCCTGAATTCCTATAGAAAGAGGTTATCCT

(b) Cloning strategy for p50ΔSP



extension containing an StuI site (plus clamping sequence) in the same frame as the StuI site at the 5' end of the intended deletion. The product of a PCR using *C9* as a target and primers 2 and 3, was cut with StuI and cloned into the target vector linearised with StuI and dephosphorylated to prevent re-ligation. Colonies resulting from this cloning were screened directly by PCR using primers 1 and 2, and bands of a size indicating an insert in the correct orientation were excised from a gel using a Geneclean II kit and subsequently digested with Bgl II and EcoR1 and cloned into pSPJCL digested with EcoR1 and Bgl II. Full length 'p50' generated by PCR primers 1 and 3 was also cloned into pSPJC2L using the same sites.

5.3.3. Construction of DNA binding deficient deletions of XrelA.

As already mentioned, a deletion of XrelA called XrelAΔSP which was predicted to be deficient in DNA binding activity, was constructed by Jill Richardson (unpublished). This clone was constructed by restriction deletion of a part of the rel homology domain between two SphI sites at 39 and 507 bp from the translational start which fortuitously re-ligate in frame (see figure 25). Construction of the double deletion, XrelAΔSP/222 from XrelAΔ222 was complicated by the presence of an additional SphI site in the vector (see map of pSP64T in Appendix 1). Unfortunately partial digestion with SphI leads to the production of a ligatable fragment of about the same size as the fragment with the desired deletion making purification by fractionation on an agarose gel impossible. To overcome this problem, XrelAΔ222 was cut to completion with ApaI, which cuts inside the desired deletion, prior to partial digestion in order to make fragments containing this region non-re-ligatable. Digested product of the predicted correct size was then isolated from an agarose gel slice as described in section 2.2.9. and re-ligated as described in section 2.2.12.

5.4. Testing the dominant negatives.

5.4.1. Inhibition of DNA binding activity of XrelA and p50.

The dominant inhibitory action of p50ΔSP, XrelAΔSP, and XrelAΔSP222 was tested in GMSA's using extracts from oocytes co-injected with mRNA encoding one of the deletion clones and either XrelA, XrelAΔ222 (used because it gives a stronger band in this assay than XrelA) or p50. The results of these experiments are shown in figure 27. It is clear from this experiment that all three dominant negatives are able to inhibit DNA binding by co-translated XrelA, XrelAΔ222 and p50, and that a ratio of 20:1 of dominant negative to p50 or XrelAΔ222 is sufficient for almost total inhibition. However, the fact that faint bands remain in the case of inhibition of XrelA binding by XrelAΔSP and the inhibition of p50 binding by p50ΔSP suggests that heterodimerisation occurs with a higher affinity than homodimerisation in both cases.

A ratio of 10:1, but not a ratio of 2:1 of XrelAΔSP or XrelAΔSP222 to XrelA is sufficient to eliminate detectable binding. The fact that a lower ratio is required for inhibition of full length XrelA binding compared to XrelAΔ222 binding may be due to the differences in DNA binding affinity between the two. XrelAΔ222 was consistently seen to bind to probe with a higher affinity than full length XrelA, which tends to show up as a diffuse band in GMSA's. The reasons for this are obscure.

These experiments show that the dominant negative clones are capable of forming inactive dimers with wild type proteins when co-translated. However, in order to inactivate protein translated prior to fertilisation a dominant negative would have to be able to displace subunits from existing dimers. As discussed in section 5.2., this protein may contribute significantly to the pool of XrelA protein which becomes localised to animal and equatorial nuclei in the blastula.

FIG. 27:- GMSA to test ability of XrelAΔSP and p50ΔSP to inhibit DNA binding by co-translated XrelA, XrelAΔ222, and p50.

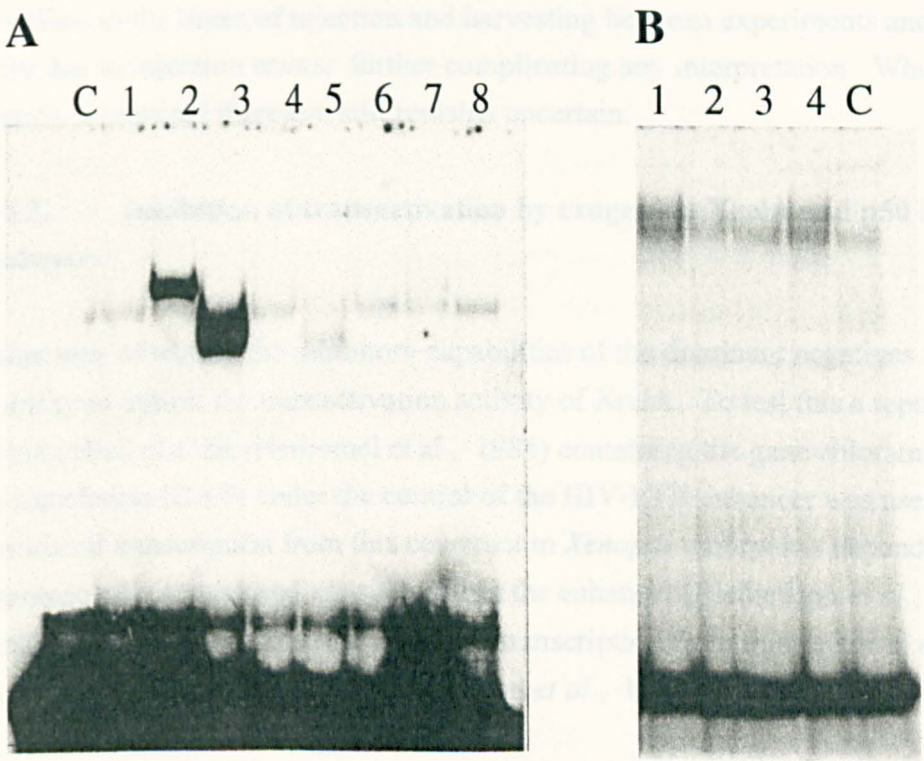
GMSA were carried out using SKBW probe with extracts from oocytes (0.5 oocyte equivalent per binding reaction) injected with mixtures of mRNA species, as indicated.

A comparison of lane A2 with A4, and lane A3 with A8 shows that at a ratio of dominant negative to DNA binding protein of 20:1, XrelAΔSP is able to completely inhibit DNA binding by p50, and p50ΔSP is able to completely inhibit DNA binding by XrelAΔ222, at least within the limitations of the sensitivity of the assay. Inhibition of XrelAΔ222 binding by XrelAΔSP, and p50 binding by p50ΔSP however is not complete at this ratio, as shown by the faint bands in lanes A5 and A7.

Inhibition of detectable binding by wild-type XrelA is seen at a ratio of 1:10 to XrelAΔSP and XrelAΔSP222. A ratio of 1:2 of XrelAΔSP to XrelA, however, does not cause detectable inhibition (lane B4).

Note the markedly stronger DNA binding affinity of XrelAΔ222 compared to full length XrelA, and the fact that XrelA binding produces a diffuse band, rather then the relatively tight one produced by the binding of XrelAΔ222.

GMSA's showing inhibition of DNA binding activity of XrelA, XrelAΔ222 and p50 by XrelAΔSP, XrelΔSP222 and p50ΔSP



GelA

C. Control (partly lost on gel)
1. 4 ng XrelAΔSP
2. 200 pg p50
3. 200 pg XrelAΔ222
4. 200 pg p50 + 4ng XrelAΔSP
5. 200pg XrelAΔ222 + 4ng XrelAΔSP
6. 4ng p50ΔSP
7. 200pg p50 + 4ng p50ΔSP
8. 200pg XrelAΔ222 + 4ng p50ΔSP

GelB

1. 800 pg XrelA
2. 800 pg XrelA + 4 ng XrelAΔSP
3. 800 pg XrelA + 4 ng XrelAΔSP222
4. 800 pg XrelA +800 pg XrelAΔSP
5. Control

To test whether the dominant negatives also have this activity oocytes were injected with wild type RNA, and then given a second injection, after 24 hours, of dominant negative RNA. Protein extracts were then made for testing by GMSA after a further 24 hours. The problem with this experiment is that the half lives of injected RNA and translated proteins are all unknown. This makes interpretation difficult. This experiment did not generate consistent results (data not shown), possibly because of variations in the times of injection and harvesting between experiments and also possibly due to injection errors, further complicating any interpretation Whether co-translation is required therefore still remains uncertain.

5.4.2. Inhibition of transactivation by exogenous XrelA and p50 in embryos.

A further way of testing the inhibitory capabilities of the dominant negatives is to test their ability to inhibit the transactivation activity of XrelA. To test this a reporter construct called pLC2R (Herbomel et al., 1984) containing the gene chloramphenicol acetyl transferase (CAT) under the control of the HIV-LTR enhancer was used. Unstimulated transcription from this construct in *Xenopus* embryos is dependent on the presence of both Sp1 and κB sites within the enhancer (Richardson *et al.*, 1994) The ability of full length XrelA to stimulate transcription from this vector in embryos has been characterised previously (Richardson *et al.*, 1994).

Batches of embryos were unilaterally injected at the two cell stage with pLC2R (250 pg/embryo) and either XrelA message alone (100 pg/embryo), or XrelA message in combination with RNA encoding one of the dominant negatives (1 ng/embryo). Embryos were checked for signs of necrosis, and healthy embryos were harvested at stage 11 as described in section 2.3.11. Extracts were then assayed for CAT activity as described in section 2.3.11. The level of radioactive product was measured using a Molecular dynamics phosphorimager, and then normalised for soluble protein. The results of this experiment are shown in figure 28. It is clear from the figure that all the dominant negatives were capable of inhibiting transactivation by exogenous XrelA. The biggest reductions in XrelA induced CAT activty were seen with XrelAΔ222. This may be because it is capable of inhibiting transactivation from this construct by endogenous factors.

The ability of dominant negatives to affect unstimulated levels of transcription from pLC2R was tested by repeating the experiment described above, but without co-injection of XrelA message. The results of this experiment are shown in figure 29.

The problem with experiments of this type is that there is considerable variation in the level of unstimulated transcription from reporter constructs measured between batches of embryos (although not in repeated assays from the same extract), even within the same experiment. This variation can be as much as +/- 25% (data not shown). This may reflect variation in replication of the injected reporter construct, and so the problem might feasably be controlled by measuring the amount of reporter DNA present by Southern blotting, and normalising the data for this. In the absence of such controls this experiment is only useful for measuring large reductions in transcription. As can be seen from figure 29, CAT activity normalised for soluble protein content in extracts from embryos injected with each of the DNA binding deficient dominant negatives fell within this error range. It is therfore not possible to conclude whether a proportion of the unstimulated transcription from pLC2R is due to rel family dimers (the expected targets of these dominant negatives). XrelAΔ222, on the other hand, was able to repeatably reduce transcription levels to around 10% of control levels. The reason for the greater efficiency of XrelAΔ222 in inhibiting unstimulated transcription from pLC2R is likely to be due to its ability to inhibit transactivation by non-rel family members by saturating κB sites in the promoter.

5.4.3. Testing dominant negatives for squelching activity.

Potentially one of the greatest drawbacks of using XrelA based dominant negatives is that they may cause general inhibition of activated transcription, known as squelching. The evidence for squelching by wild-type XrelA is discussed in the introduction (section 1.1.7.). Given that there is compelling evidence for squelching by XrelA, particularly when expressed at high levels, it might seem likely that XrelA ΔSP, which after all contains the complete transactivation domain as well as the putative nuclear localisation signal, would have the same activity. That this is not a forgone conclusion is illustrated by some work on squelching by serum response factor (SRF). Like relA, SRF is able to squelch transactivation by acidic transactivators

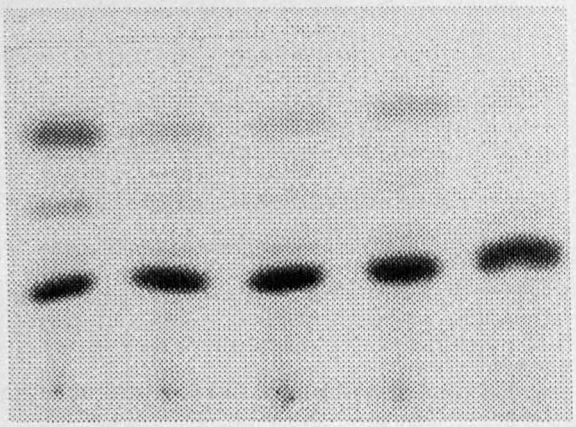
FIG. 28:- Dominant negative inhibition of transactivation by exogenous XrelA in embryos.

Embryos were injected with a mixture of RNA's as indicated, plus the HIV-LTR based reporter construct pLC2R (250 pg/embryo). Protein extracts were made from these embryos at stage 11, and assayed for CAT activity, and soluble protein concentration.
(A) Shows the radioactive product, visualised by autoradiography from a TLC plate (upper spot).
(B) Shows a histogram of CAT activity, as assayed using a phosphorimager followed by quantification of reaction product using ImageQuant and normalisation to soluble protein Activity is expressed as a percentage of XrelA induced activity.

Note that all dominant negatives are able to inhibit transactivation by exogenous XrelA. Inhibition by XrelAΔ222 is most complete, presumably because it is acting by saturating binding sites on the reporter construct with non-transactivating homodimers.

Inhibition of XrelA κB transactivating activity by dominant negative interference clones.

A



1. XrelA
2. XrelA + XrelAΔSP
3. XrelA + p50ΔSP
4. XrelA + XrelAΔSP222
5. XrelA + XrelAΔ222

Ratio of XrelA to dominant negative RNA = 1:10 (100 pg : 1 ng)

Reporter construct - pLC2R

B

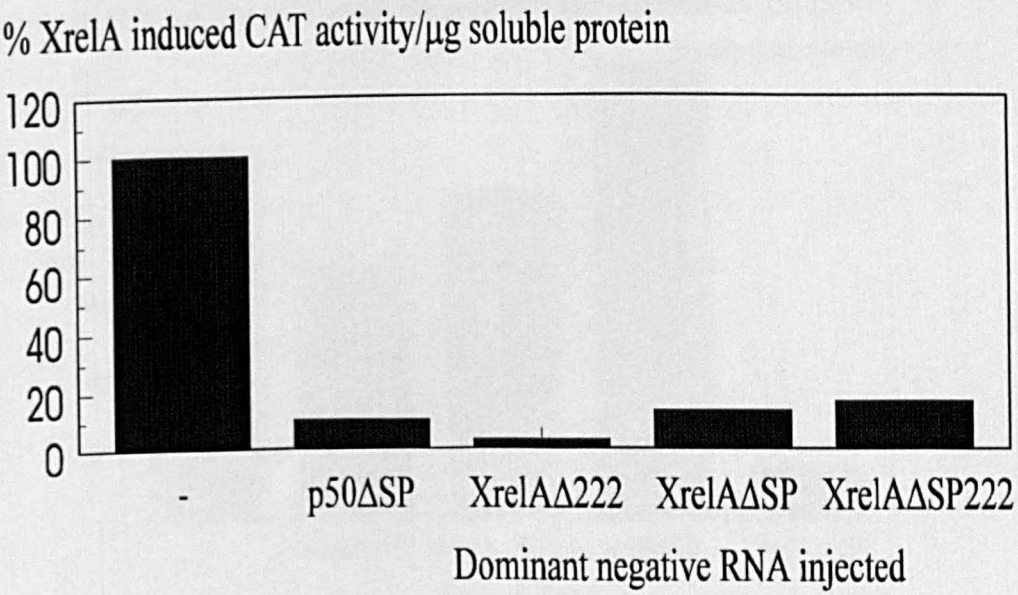


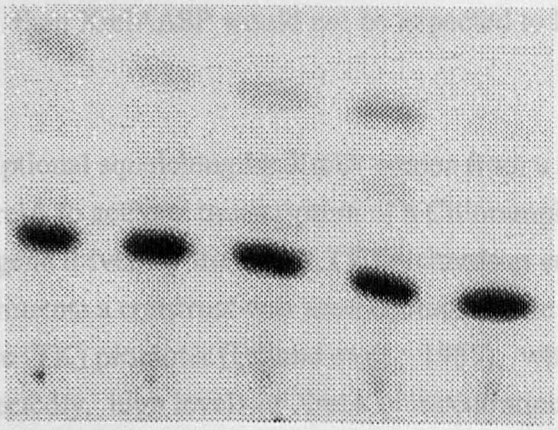
FIG. 29:- Experiment to test the ability of domianant negatives to inhibit endogenous kB transactivating activity at stage 11.

Embryos were injected with a mixture of RNA's as indicated, plus the HIV-LTR based reporter construct pLC2R (250 pg/embryo). Protein extracts were made from these embryos at stage 11, and assayed for CAT activity and soluble protein concentration..
(A) Shows the radioactive product, visualised by autoradiography from a TLC plate (upper spot).
(B) Shows a histogram of CAT activity, as assayed using a phosphorimager followed by quantification of reaction product using ImageQuant, and normalisation to soluble protein. Activity is expressed as a percentage of XrelA induced activity.

Experimental errors were very high in this experiment. Variation in CAT activity in extracts from uninjected embryos, even within the same experiment, varied over a range of approximately +/- 25%. However, very little variation was seen when CAT activity was measured in the same sample more than once (data not shown). It therefore seems likely that the variation is due to differences in repilication of the injected reporter construct. CAT activity in extracts from embryos expressing the DNA binding deficient domainant negatives all fell within this error range. XrelAΔ222, on the other hand, was able to repeatably lead to reductions in CAT activity to around 10% of control levels. It seems likely that this reduction is due to XrelAΔ222 saturating binding sites on the reporter construct, and so inhibiting all κB dependent transactivation, and not just that due to rel family members.

Experiment to test the ability of dominant negatives to inhibit endogenous κB dependent transactivation at stage 11.

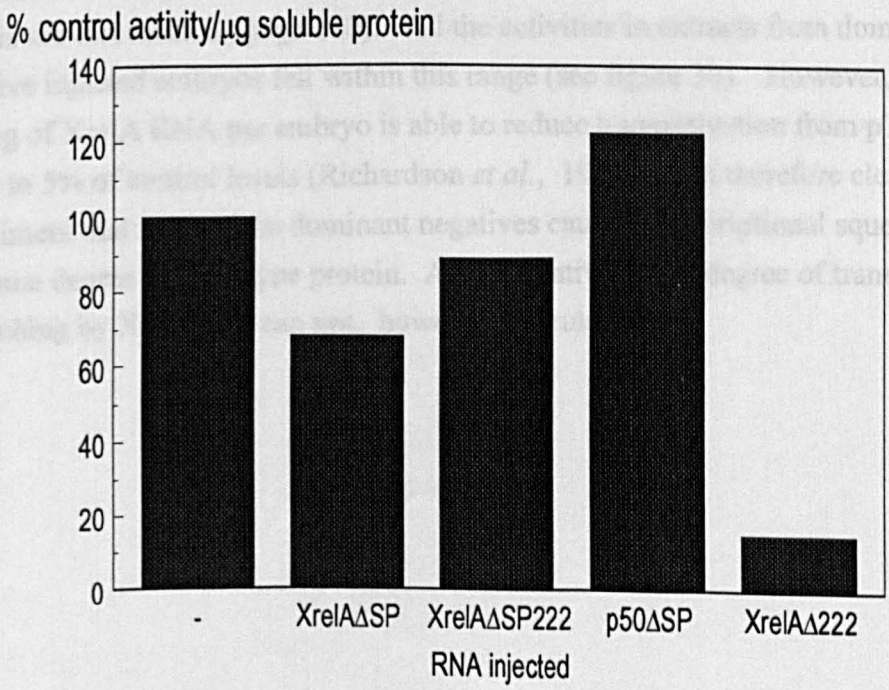
A



1. Control
2. XrelAΔSP
3. XrelAΔSP222
4. p50ΔSP
5. XrelAΔ222

Reporter construct - pLC2R (250 pg / embryo)
RNA injected - 1 ng / embryo

B



such as VP16, but this activity is lost in a range of mutants lacking DNA binding activity (Prywes and Zhu, 1992). The same has also been shown to be true of the squelching activity of a chimeric protein consisting of the VP16 transactivation domain fused to the human oestrogen receptor (Gilbert *et al.*, 1993). If DNA binding activity is a general requirement for transcriptional squelching (I have not found any published evidence to the contrary), then XrelAΔSP would not be expected to have squelching activity.

Further evidence for transcriptional squelching by XrelA comes from a set of reporter assay experiments using XrelA to activate transcription of a Chloramphenicol Acetyl Transferase (CAT) reporter gene driven by the HIV LTR (Richardson *et al.*, 1994). As a control for these experiments a construct was used containing the CAT gene driven by the thymidine Kinase (TK) promoter (Edlund *et al.*, 1985), which does not contain a κB site. Unexpectedly, high levels of XrelA (concentrations above 2 ng/embryo) were shown to dramatically reduce levels of transcription from this construct. It seemed likely, therefore that the TK/CAT construct could provide a simple test for the transcriptional squelching ability of various deletion clones of XrelA.

To test this, mRNA encoding each of the dominant negative interference clones (approximately 2 ng/embryo) was individually co-injected with pTKCAT in to two cell embryos. These embryos were harvested at stage 11 and the resulting extracts were assayed for soluble protein concentration and CAT activity as described in sections 2.3.3. and 2.3.11. respectively. As with the pLC2R reporter there was a variation in activity between control extracts of around +/- 25% (the possible reasons for this are discussed on page 110). All the activities in extracts from dominant negative injected embryos fell within this range (see figure 30). However, injection of 2 ng of XrelA RNA per embryo is able to reduce transactivation from pTKCAT down to 5% of control levels (Richardson *et al.*, 1994). It is therefore clear from this experiment that none of the dominant negatives causes transcriptional squelching to the same degree as wild-type protein. A comparatively low degree of transcriptional squelching by XrelAΔSP can not, however, be ruled out.

FIG. 30:- Experiment to test transcriptional squelching by dominant negatives.

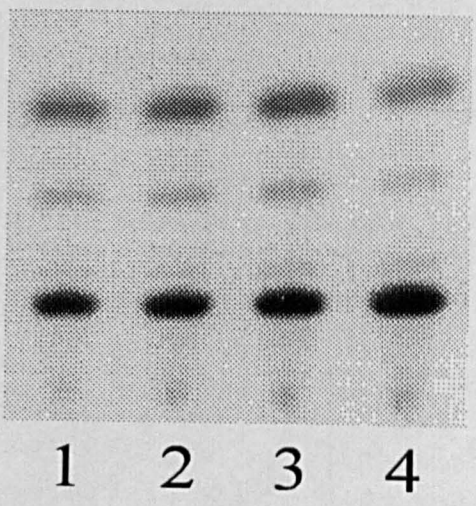
Embryos were injected with a mixture of RNA's as indicated, plus the HIV-LTR based reporter construct pLC2R (250 pg/embryo). Protein extracts were made from these embryos at stage 11, and assayed for CAT activity.
(A) Shows the radioactive product, visualised by autoradiography from a TLC plate (upper spot).
(B) Shows a histogram of CAT activity, as assayed on a phosphorimager followed by quantification of reaction product using ImageQuant. Activity is expressed as a percentage of XrelA induced activity.

As with the pLC2R reporter there was a variation in activity between control extracts of around +/- 25% (the possible reasons for this are discussed on page 110). All the activities in extracts from dominant negative injected embryos fell within this range. However, injection of 2 ng of XrelA RNAper embryo is able to reduce transactivation from pTKCAT down to 5% of control levels (Richardson *et al.*, 1994). It is therefore clear from this experiment that none of the dominant negatives causes transcriptional squelching to the same degree as wild-type protein.

Experiment to test for transcriptional squelching by dominant negatives.

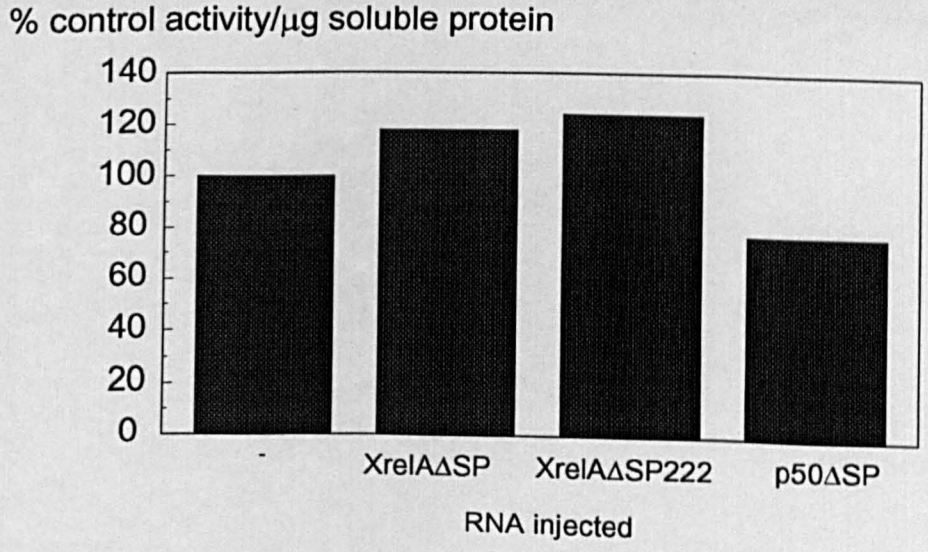
A

1. Control
2. XrelAΔSP
3. XrelAΔSP222
4. p50ΔSP



Reporter construct - pTKCAT (250 pg / embryo)
RNA injected - 1 ng / embryo

B



5.4.4. Effects of dominant negatives on endogenous κB binding activities.

As the range of specificity of the DNA binding deficient dominant negatives would be expected to include complexes containing other rel family members, it is possible that κB binding complexes which do not contain XrelA will be sensitive to their presence. Mapping the distribution of such activities may help in the interpretation of dominant negative phenotypes.

To test this embryos were bilaterally injected with either 4ng or 400 pg of mRNA encoding XrelAΔSP or p50ΔSP at the two cell stage.. Embryos were harvested at stage 13, a stage at which all the specific binding activities previously characterised (see section) can be detected, and the extracts used for GMSA's with SKBW probe (as described in section 2.3.1.). The results of this experiment are shown in figure 31. In the experiment shown, binding of the fastest migrating κB binding complex appeared to be inhibited, or much reduced, by 4 ng injections of either of the two RNA's. However, this result was not repeatable, and is probably an artefact of the instability of this complex noted previously (see section 4.2.3.). What was repeatable, however, was the effect of p50ΔSP on the slowest migrating of the three κB binding complexes. At the lower concentration of injection (400 pg/embryo) binding by this complex was inhibited, and the intensity of the non-specifc band above it was slightly enhanced. At higher concentrations (4 ng/embryo), binding also appears to be inhibited, and a strongly binding complex was seen just above. This strong band could represent a separate effect to the inhibition, or could result from shifting and enhancement of the lower specific band. It should also be borne in mind that this (these) effect(s) may not be direct. For example, the enhanced band could be an upregulated gene product.

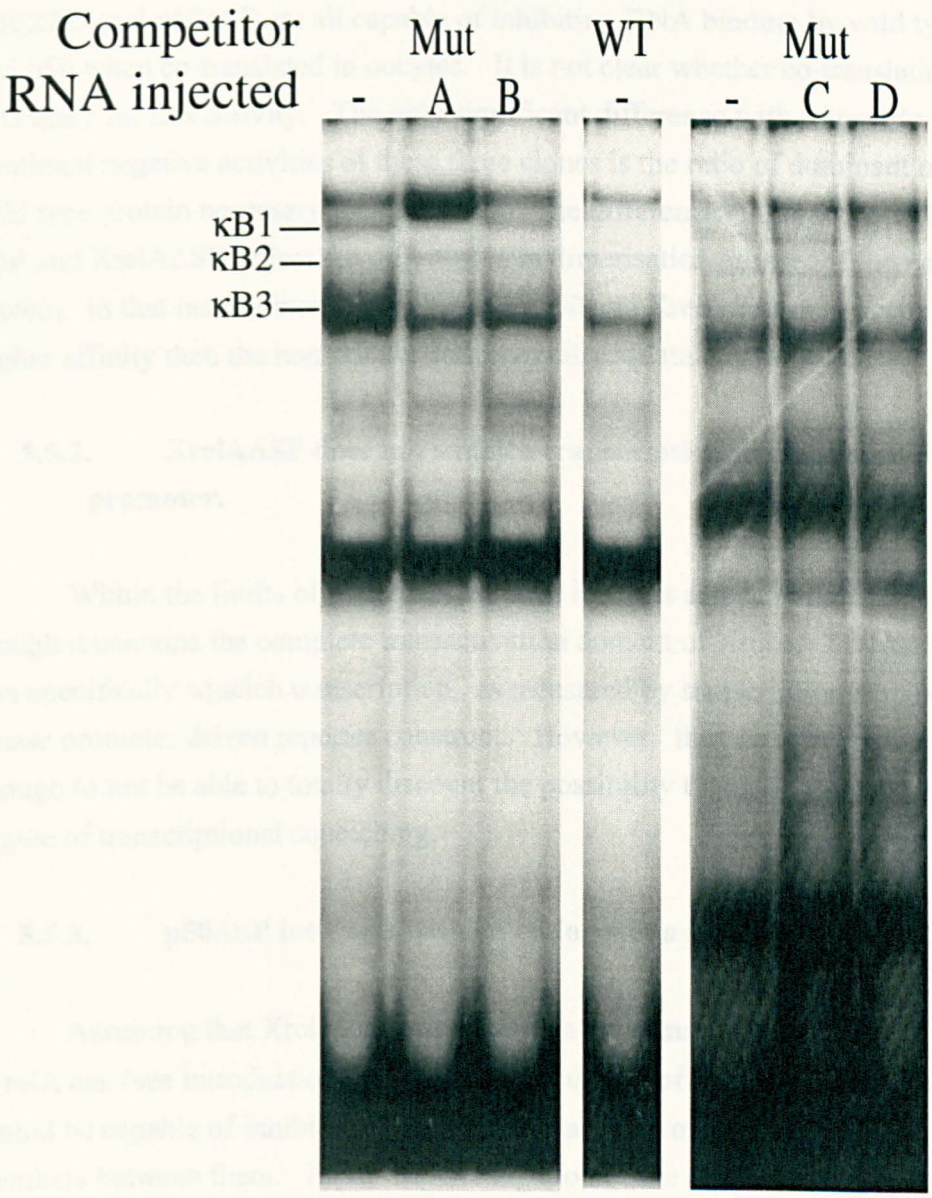
FIG. 31:- GMSA's showing effect of expression of DNA binding deficient dominant negatives on endogenous κB binding complexes.

Embryos were bilaterally injected with RNA, as indicated and harvested at stage 13 (a stage at which all the characterised binding activities are present) for gel mobility shift assays. All binding reactions were carried out with SKBW probe in the presence of 100 fold excess of mutant competitor, except in one case where wild-type competitor was used. The three bands lacking in this lane compared with the control lanes are the three specific binding activities, known as κB1, κB2 and κB3 described in section 4.2.3.

As discussed in this section, κB3 is unstable, leading to significant variation in its relative level of binding (compared to other bands) between extracts from the same stage. The variation seen in this experiment may therefore be artifactual. This is supported by the finding that the apparent inhibition seen here with high levels of XrelA RNA injection (4ng) was not repeatable.

A result which was repeatable is the inhibition and/or interaction of p50 ΔSP with the κB1 complex. This activity was not present in extracts from embryos injected with 400 pg of p50ΔSP RNA, and the (non-specific (?)) band above appeared to be slightly enhanced. In extracts from embryos injected with higher levels of this RNA (4ng), κB1 activity also seems to be lost, but the upper band is significantly enhanced. This result is difficult to interpret. One possibilty is that some interaction with p50ΔSP is shifting the complex, rather than inhibiting its activity. On the other hand the enhanced band could represent a completely different phenomenon.

GMSA showing effect of dominant negatives on endogenous κB binding activites in embryos.



RNA injected:-
A = 4 ng p50ΔSP
B = 4ng XrelAΔSP
C = 400 pg XrelAΔSP
D = 400 pg p50ΔSP

Competitors:-
Mut = Mutant
WT = Wild-Type

κB1, 2 & 3 are specific binding activities

5.5. Conclusions and discussion.

5.5.1. All three DNA-binding deficient deletions have dominant negative activity.

It is clear from the experiments detailed in this chapter that XrelAΔSP, XrelA ΔSP222, and p50ΔSP are all capable of inhibiting DNA binding by wild type XrelA and p50 when co-translated in oocytes. It is not clear whether co-translation is necessary for this activity. The only significant difference with respect to the dominant negative activities of these three clones is the ratio of dominant negative to wild type protein necessary for inhibition. The differences in this respect between p50 ΔSP and XrelAΔSP reflect the differences in dimerisation affinity of the wild type protein, in that heterodimerisation between p50 and XrelA seems to occur with a higher affinity than the homodimerisation of either (data not shown).

5.5.2. XrelAΔSP does not squelch transcription from a thymidine kinase promoter.

Within the limits of experimental error it seems safe to conclude that, even though it contains the complete transactivation domain of XrelA, XrelAΔSP does not non-specifically squelch transcription, as measured by transcription from a thymidine kinase promoter driven reporter construct. However, that experimental error is large enough to not be able to totally discount the possibility that it is capable of a small degree of transcriptional squelching.

5.5.3. p50ΔSP interacts with an endogenous κB binding complex.

Assuming that XrelA can dimerise with the same range of rel family members as relA can (see introduction, page 14 for discussion of this), XrelAΔSP and p50ΔSP should be capable of inhibiting DNA binding activity of all the known rel family members between them. From the investigation of the effects of the dominant negatives on specific κB binding activities it therefore seems safe to conclude that the two faster migrating, unaffected activities do not involve rel family members, or at least not rel family members with activities like those currently characterised.

p50ΔSP, but not XrelAΔSP, is capable of inhibiting DNA binding by the specific κB binding complex κB3 in embryos, although it is by no means certain that this is a direct effect. It may be possible to test whether the inhibition requires protein

synthesis by incubating injected embryos with cycloheximide. The only characterised rel family member which will heterodimerise with p50 and not with p65 is relB suggesting that if the inhibition does involve direct interaction of p50ΔSP with a component of the binding complex then a relB homologue is a possible candidate for this component.

6. Analysis of phenotypes

6.1. Introduction

The main reason for constructing dominant negative interference clones was, of course, to study the effects of their expression on development. This chapter deals with the descriptive analysis of phenotypes resulting from the injection of RNA encoding the various DNA binding deficient dominant negative clones at the two cell stage.

6.2. Results

The phenotypes due to expression of the three dominant negative clones in whole embryos were investigated by bilateral[18] injection of a range of concentrations of mRNA at the two cells stage. Embryos were observed and scored throughout development, and any embryos showing signs of necrosis were discarded to avoid artifactual phenotypes. Selected embryos were wax embedded, sectioned and stained as described in section 2.5. For every concentration of RNA injected a range of phenotypes can be observed. This is most likely due, at least in part, to differences in the distribution of injected RNA. In order to correlate phenotypic effects with RNA distribution, experiments were carried out involving co-injection of the dominant negative RNA with RNA encoding β-galactosidase fused to a nuclear localisation signal (nuc.β-gal; see appendix 2 for details). The distribution of this gene product is easily assayed by staining with X-gal, as described in section 2.3.10. Such experiments, have confirmed that the distribution of injected RNA is highly variable. This is illustrated by the experiment shown in figure 37.

6.2.1. Expression of p50ΔSP in embryos has no obvious phenotypic effects.

Expression of p50ΔSP in embryos had no phenotypic effects, as judged externally or by histology, except at very high concentrations of injected RNA (approximately 5ng) which produced occasional and inconsistent defects (data not

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18 'Bilateral' injection is possible at this stage because the first cleavage plane defines the plane of bilateral symmetry in most embryos (see introduction, page 29)

shown). As inhibition of DNA binding by XrelA can be inhibited by only a 20 fold excess of p50ΔSP (see figure 27), such massively high levels of expression should not be needed to inhibit DNA binding by endogenous XrelA. However, it is important to note that, as it is unclear whether co-translation is necessary for this inhibitory effect, there may be a pool of maternal XrelA protein (and/or other rel family member) whose activity remains unaffected by this dominant negative

6.2.2. The phenotype due to XrelAΔSP has some similarities to that due to the dominant negative FGF receptor XFD.

Unlike p50ΔSP, XrelAΔSP injection produces quite dramatic phenotypic effects, although these are only seen when 200 pg or more RNA is injected into each embryo. Unless otherwise stated, all the phenotypes described here resulted from injection of approximately 1 ng of mRNA per embryo. As with all experiments of this type there is a certain amount of phenotypic variation, but the phenotype can basically be summarised as follows. All injected embryos exhibited shortening of the trunk (to varying degrees), accompanied by ventral 'enlargement', and frequently by upward curving of the trunk. A proportion of injected embryos showed splitting of dorsal structures of the trunk behind the head around an exposed yolk plug. This was always accompanied by loss of an eye on one side of the head and in a minority of cases loss of eyes on both sides. If allowed to develop, almost all injected embryos showed an inhibition of tail extension. A typical distribution of these defects resulting from injections of approximately 1ng of RNA per embryo is illustrated by the experiment scored in figure 34.

Figure 32 shows a typical embryo at control stage 42, from a batch bilaterally injected with approximately 1ng of XrelAΔSP mRNA (control shown above). One of the most striking things about this phenotype is its similarity, at least externally, to that caused by expression of the dominant negative FGF receptor, XFD (see introduction, page 42). Both phenotypes usually involve apparent splitting of dorsal structures of the trunk around a still open yolk plug, usually immediately behind the head. In some cases, and especially at lower concentrations of injected RNA, this

FIG. 32:- Comparison of control (above) with XrelAΔSP injected embryo at stage 42.

The embryo shown is representative of the majority of embryos from a batch injected with 1ng of XrelAΔSP RNA per embryo. Note reduction in axis extension and inhibition of tail elongation. Most embryos also have an open yolk plug behind the head, although this is difficult to see in this embryo. Most injected embryos are stage retarded. This can be seen in this case by the immaturity of head structues compared to the control.

Scoring for inhibition of tail extension:-

15/24 - tail extension largely or completely inhibited

9/24 - tail extension occurs relatively normally (tails are generally shorter than controls, but this is probably due to stage retardation).

FIG. 33:- Comparison of XFD and XrelAΔSP injected embryos at control stage 15.

Embryos were injected bilaterally at the two cell stage with XrelAΔSP RNA (1 ng per embryo) and fixed at control stage 15 for scoring and photography. Note - photograph (B) was provided by Caroline Beck, and is shown for the purposes of comparison with the XrelAΔSP injected embryo. It shows an embryo at control stage 15 which had been bilaterally injected with XFD mRNA at the two cell stage.

Scoring:-

1ng XrelAΔSP:-

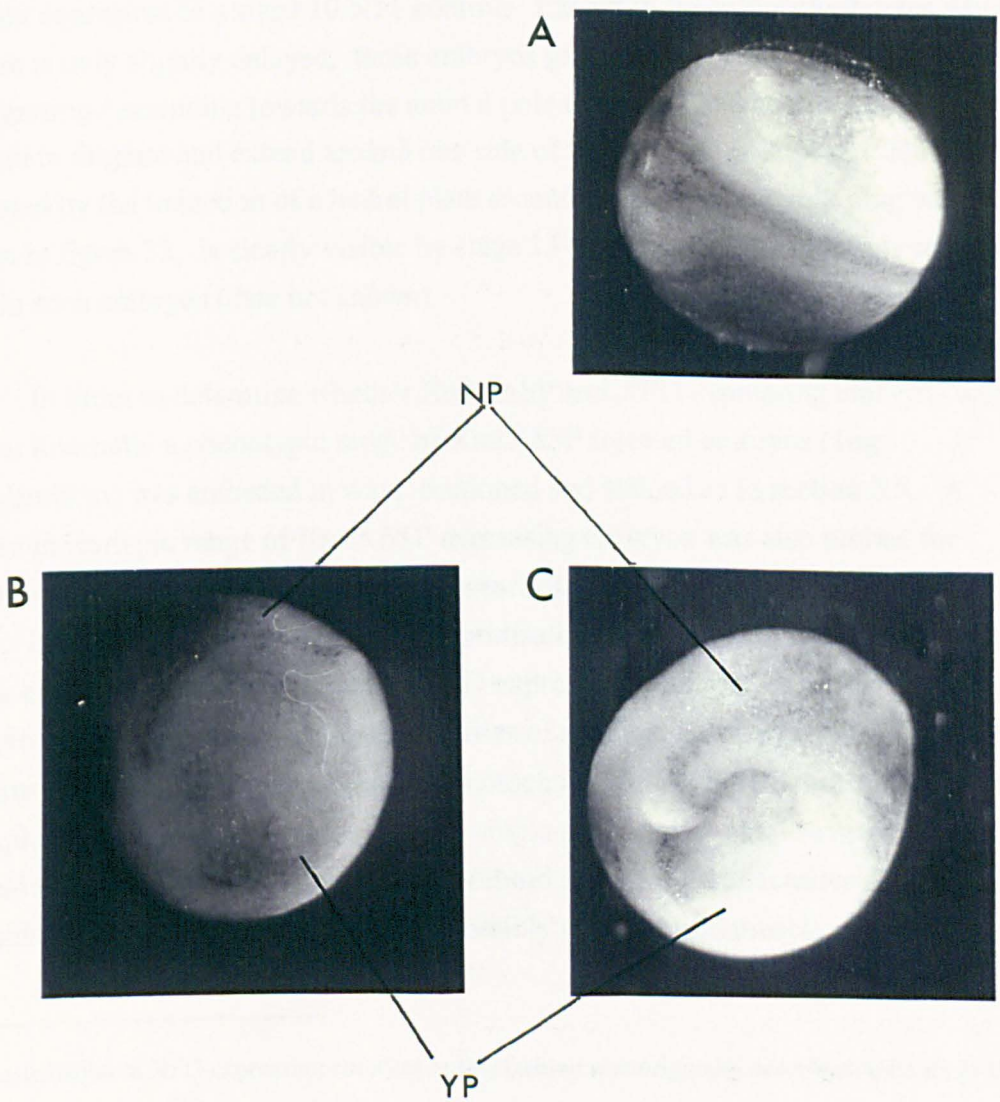
16/29 - Have large exposed yolk plug still visible; induced neural plate shows migration/extension of the dorsal mesoderm is more lateral than animal.

7/29 - Have small exposed yolk plug visible, induced neural plate shows that migration/extension of the dorsal mesoderm is more animal than lateral.

6/29 - Blastopore has closed but, due to stage retardation, embryos look like normal controls of about stage 13.

In both XFD (B) and XrelAΔSP (C) injected embryos epiboly is retarded, leaving an exposed yolk plug (YP) still visible at control stage 15 (control shown in (A)). Dorsal mesoderm migrates around the yolk plug in these embryos instead of towards the anima pole, as in controls, as evidenced by the induced neural plate (NP) around one side of the yolk plug.

Note - the embryo shown in (C) is not the most extreme example of this phenomenon seen with XrelAΔSP.


A
NP
B
C
YP

splitting does not occur. This has also been seen in embryos expressing XFD (Amaya *et al.*, 1991), and seems to correlate with ventral, but not dorsal, targeting of RNA (Isaacs *et al.*, 1994). In both XFD and XrelAΔSP expressing embryos the axes which do form are severely shortened, suggesting an inhibition of convergent extension. Both phenotypes also involve an inhibition of tail extension. The head defects seen in some XrelAΔSP expressing embryos (see below) do not fit with the descriptions found in the literature of the XFD phenotype. However head defects in XFD expressing embryos are mentioned as unpublished observations in a recent publication (Cornell *et al.*, 1995)), and have been observed by other members of the research group (C. Beck, per. comm.).

The similarity of the two phenotypes is also very strong during the gastrula and neurula stages. In both cases blastopore closure is inhibited, or in some cases delayed until the neurula stages. In the most extreme the extent of blastopore closure is roughly equivalent to a stage 10.5/11 control. Except in the minority of cases where closure is only slightly delayed, these embryos gastrulate very eccentrically. Instead of migrating / extending towards the animal pole the dorsal and anterior mesoderm appears to migrate and extend around one side of the exposed yolk plug. This is indicated by the induction of a neural plate around one side of the yolk plug which, as shown in figure 33, is clearly visible by stage 15[19]. Blastocoel retention is commonly seen in such embryos (data not shown).

In order to determine whether XrelAΔSP and XFD expressing embryos were similar internally a phenotypic range of XrelAΔSP injected embryos (1ng RNA/embryo) was embeded in wax, sectioned and stained as in section 2.5. A similar phenotypic range of XrelAΔSP expressing embryos was also probed for expression of Collagen II, a marker for notochord, somites and otic vesicles (Amaya *et al.*, 1993), by whole mount *in situ* hybridisation as described in section 2.2.18. These experiments show that, unlike XFD expressing embryos, XrelAΔSP expressing embryos form notochord and segmented files of somites, even when the yolk plug remains exposed. In these instances the notochord which forms is not split around the yolk plug. Instead these embryos have a single set of dorsal axial structures split around the open yolk plug so that the notochord and one file of somites are found on one side, with more staining material, possibly disorganised muscle, forming on the

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19 Gastrulation of XFD expressing embryos in this fashion was originally described in Issacs et al., 1994.

FIG. 34:- Collagen II expression in XrelAΔSP injected embryos at control stage 32.

Heterozygous albino embryos (albino♀ x pigmented ♂) bilateraly injected with approximately 1 ng of XrelAΔSP mRNA per embryo were fixed at control stage 32 and stained for the expression of collagen II by *in situ* hybridisation.

Scoring.

Group 1 (11/23):- Shortened trunk but otherwise relatively normal, although almost all of these (9/12) have notochord over to one side (as in figure 35). Head defects were rare in this group, but some of the more severely laterally asymmetric had eyes on the notochord side only (again as in figure 35)

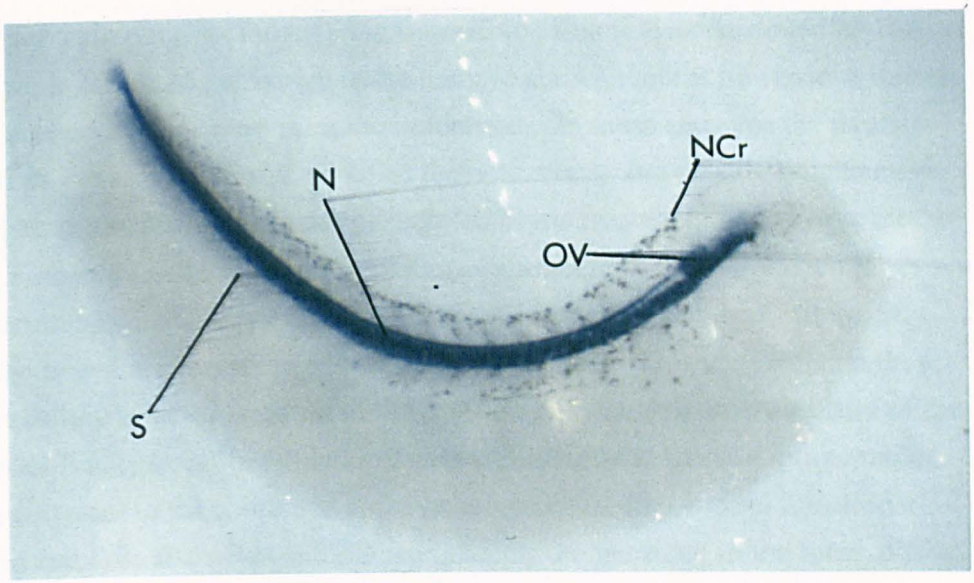
Group 2 (12/23):- Shortened trunk split dorsally around open yolk plug with dorsal axial tissue split as in (**C**). Of these most had no eye on the other side to the notochord, but 3/12 also showed loss of both eyes.

A. Control (cleared)
Note staining in notochord (**N**), otic vesicle (**OV**), and also faint staining in the somites. The only other pigment visible (zygotic contribution) is in the eye, and in migrating neural crest cells (**NCr**) which can be seen around the somite.

B. Mildly affected Group 1 type embryos. These embryos are relatively normal apart from having shortened trunks. The two embryos shown are not laterally asymmetric.

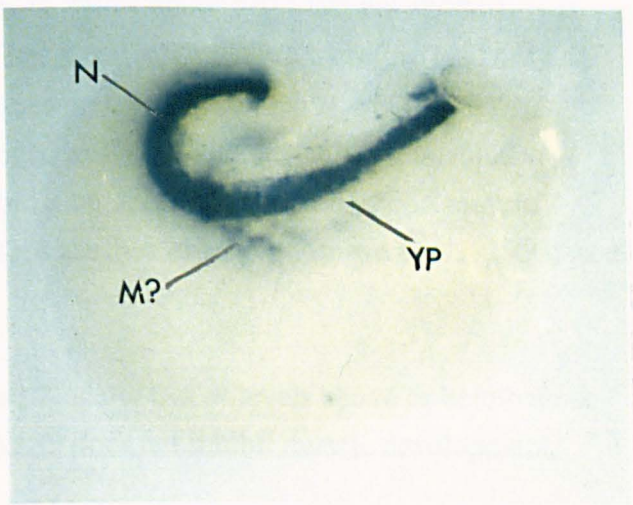
C. Ventral / lateral view of typical group 2 embryo.
Note the splitting of dorsal axial tissues around the exposed yolk plug behind the head with the notochord (N)on one side of the yolk plug (YP), and disorganised staining material (possibly muscle(M?) on the other side.

A



B

C



other side of the yolk plug, as indicated in figure 34. This is also illustrated by the sections shown in figure 36, although in the embryo shown there is no obvious muscle on the other side of the yolk plug from the notochord. In these embryos the neural tube is found on the same side of the split as the notochord, and usually fails to close completely (see figure 36). The heads of these embryos frequently lack an eye on the side lacking notochord and occasionally lack eyes altogether. The Collagen II staining of embryos which do not initially appear to have this 'split axis', frequently shows that the notochord is over to one side (see figure 35). This suggests that they may be more mildly affected versions of the 'split axis' phenotype where closure of the blastopore does finally occur but only after gastrulation around the yolk plug, rather than being equivalent to the shortened axis phenotype caused by ventral injection of XFD. These embryos also often exhibit head defects on one side, in the form of loss of one eye.

In most of the histologically examined XrelAΔSP injected embryos the dorsal trunk structures are similar in proportion to those in controls. The endoderm, on the other hand, is much larger in cross section (see figure 36). This is especially true anteriorly, as illustrated by the embryo in figure 35C.

Although even the most extreme embryos have notochord and somites they seem to have reduced amounts of these tissues compared to controls. In order to test whether the lack of a complete elimination of these tissues might be due to (enough) injected RNA not reaching them embryos were co-injected with XrelAΔSP and RNA encoding nuclear β-galactosidase (nuc.β-gal. (10 pg/embryo). These embryos were fixed and stained for β-galactosidase activity as detailed in section 2.3.10., and then re-fixed overnight in MEMFA before staining for collagen II expression by *in situ* hybridisation. Dilution series experiments with injected nuc.β-gal. RNA show that the cut off point for detectable β-galactosidase levels of expression is quite sharp (activity is not detectable at injection levels below about 5pg (data not shown). Co-injection of such low concentrations of nuc.β-gal RNA should therefore lead only to staining of cells that have received a relatively high dose of RNA. Similar experiments with XFD injected embryos have shown that, even when injected at concentrations which do not cause gastrulation defects, XFD can inhibit muscle differentiation as long as the injected RNA reaches its target (Amaya *et al.*, 1993; see introduction, page 42).

The experiments shown in figure 37 show that at levels above or below those which cause gastrulation defects XrelAΔSP does not inhibit muscle development,

even when the highest levels of RNA, as indicated by X-gal staining, are present in the somites as in the two embryos shown.

6.2.3. A variant of XrelAΔSP lacking the transactivation domain has no obvious phenotypic effects.

The fact that the phenotype observed in XrelAΔSP expressing embryos is not also caused by p50ΔSP, even though this clone is equally effective at inhibiting DNA binding by wild type protein suggests that XrelAΔSP exerts its phenotypic effects by some mechanism other than a dominant inhibitory action against XrelA. This interaction could take one of two forms. RelA has recently been shown to interact with a number of different transcription factors (discussed in detail in the final chapter; see page 151). It seems plausible to suggest that XrelAΔSP might be acting by sequestering some developmentally important transcription factor. The other form that this interaction could take is the sequestering of an essential co-factor for acidic transactivation, i.e.:- squelching.

In order to test whether the transactivation domain was necessary for the phenotypic effects of XrelAΔSP, a deletion clone called XrelA ΔSP222 lacking both the putative DNA binding and transactivation domains was constructed (see chapter 5, sections 5.2. and 5.3.3.) and shown to be able to act as a dominant negative (see figure 25). Expression of this clone in embryos had no obvious phenotypic effect (data not shown).

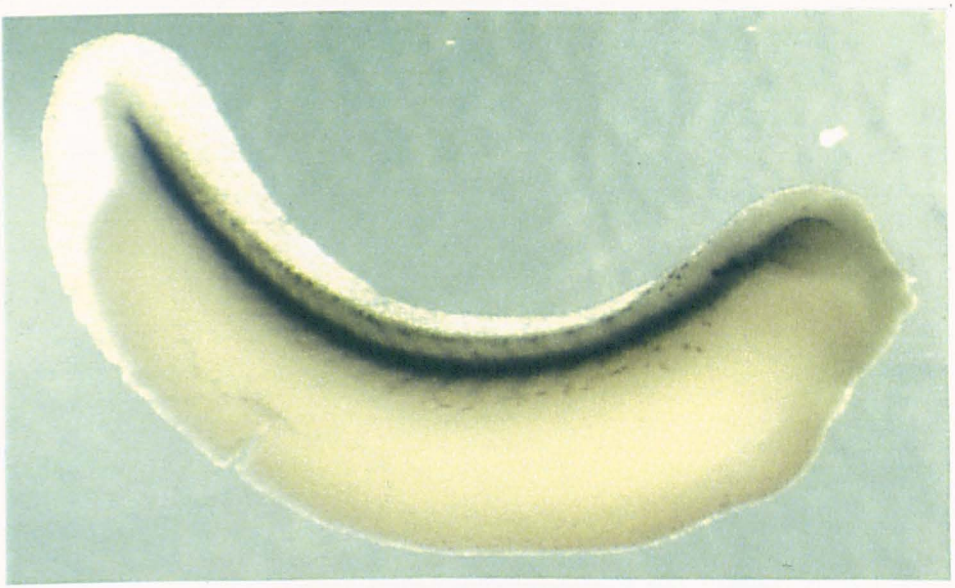
FIG. 35:- Most 'non-split-axis' embryos are laterally asymmetric

Heterozygous albino embryos (albino ♀ x pigmented ♂) bilaterally injected with approximately 2ng of XrelAΔSP RNA were fixed at control stage 32 and stained for the expression of collagen II by *in situ* hybridisation. For scoring see legend of figure 34.

A. Control.
Note clear staining of somites.

B. and C. Extreme laterally asymmetric embryo from group 1 seen from both sides. Note that the notochord and somites are clearly visible from one side only and only the notochord side has an eye. This embryo also has an upwardly curving trunk which is commonly seen in these embryos, and may result from differences in the amount of convergent extension occurring in the different germ layers.

A



B

C

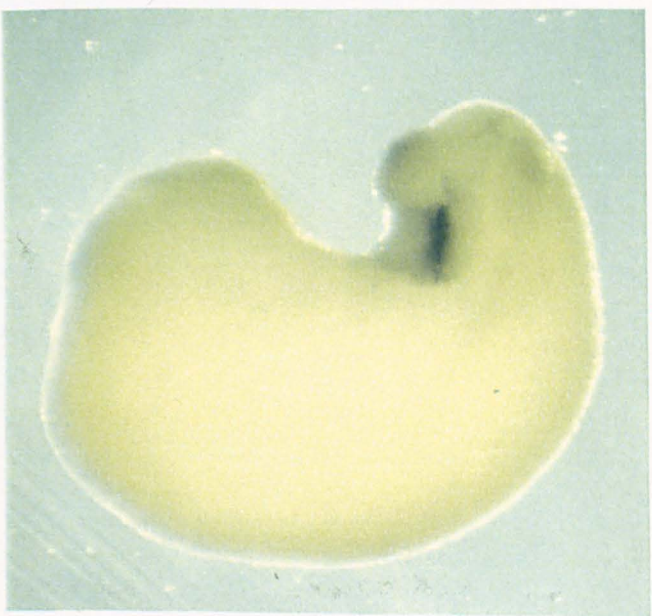


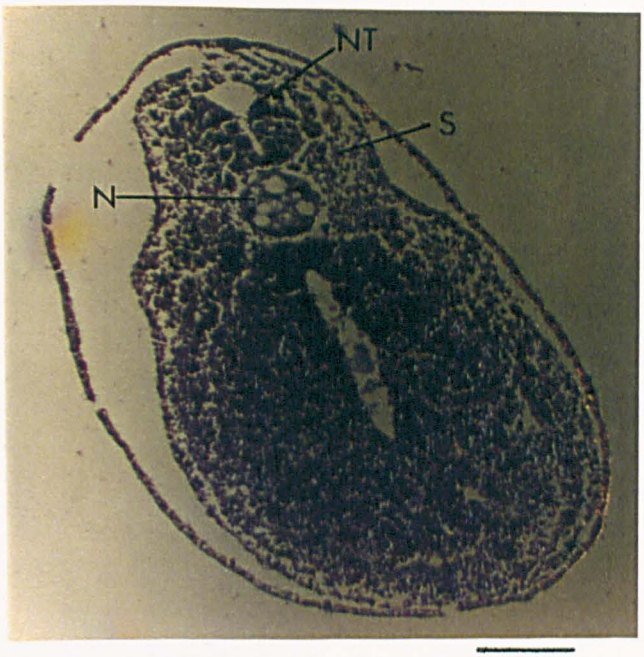
FIG. 36:- Histological analysis of XrelAΔSP expressing embryos.

Embryos bilateraly injected with a total of approximately 1ng of XrelA ΔSP RNA were fixed in MEMFA at control stage 32, embedded in wax, sectioned and stained. All sections were photographed using a 10 x objective so direct comparisons of scale can be made between the sections shown.
Scale bar = 100 μm

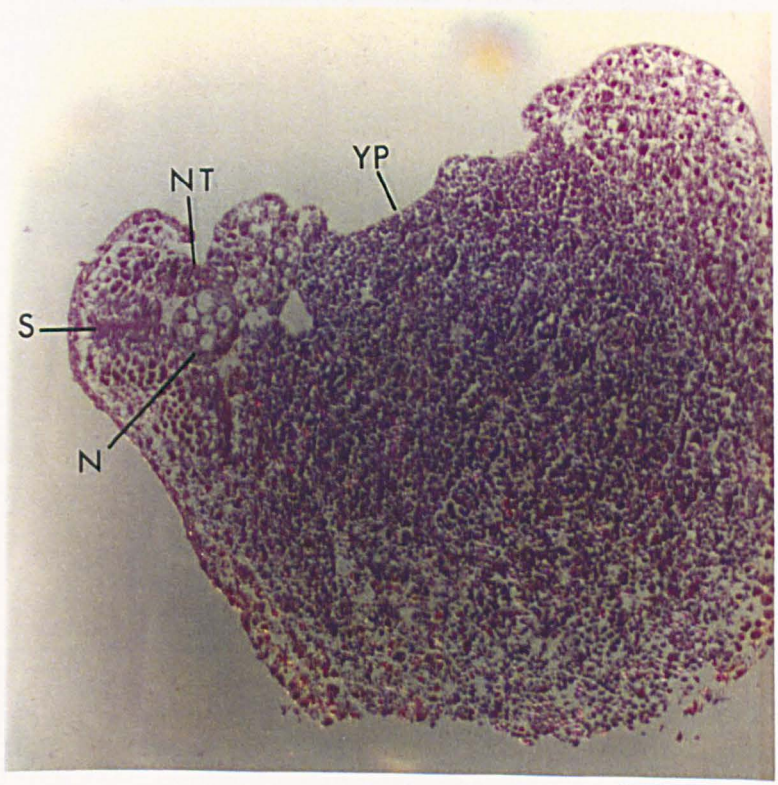
A. Mid-trunk section through a control embryo.
Note detachment of the epidermis is a common artefact of the fixation / embedding procedure used.

B. Transverse section through the mid-trunk of an XrelAΔSP 'split-trunk' embryo.
Note the large open yolk plug (**YP**) with somites (**S**) and a notochord (**N**) on one side. Owing to the curvature of the trunk (see embryo in figure 35B and C) a pair of somites can be seen in longitudinal section, rather than a single somite in transverse section as in the control. On the same side a small, open neural tube (**NT**) can be seen above the notochord. On the other side of the yolk plug there are no obvious signs of muscle at this point, just what looks like a very thick layer of lateral plate mesoderm. The other main feature of note is how broad the endoderm and lateral plate mesoderm are in comparison with the control.. By comparison, the dorsal structures remain relatively similar in proportion to the controls.

A



B



C. More anterior trunk section from the same embryo as in (B). This section is anterior to the point where the dorsal structures split around the yolk plug. Somites are still only clearly visible to one side of the notochord, and the neural tube is still open. The large amount of lateral plate mesoderm on the right side of the embryo is even more striking in this section than in (B).

C

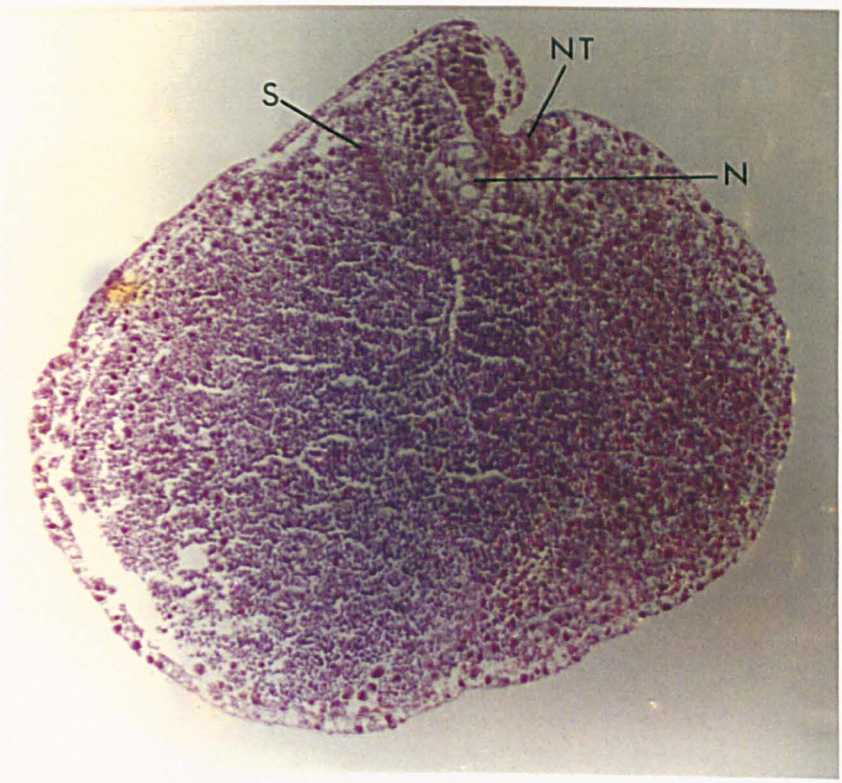


FIG. 37:- Experiment to test ability of XrelAΔSP to inhibit muscle differentiation, even when embryos gastrulate completely.

Heterozygous albino embryos (albino ♀ x pigmented ♂) bilaterally co-injected with nuc.β-gal RNA (10 pg/embryo) and XrelAΔSP RNA (as detailed below) were fixed at control stage 32 and stained for β-galactosidase activity with X-gal. They were then re-fixed overnight and stained for the expression of collagen II by *in situ* hybridisation. Collagen II expression shows up as a dark blue / purple stain in these pictures, whereas X-Gal staining is pale blue.

A. Control (stage 32) showing strong staining for collagen II in the notochord and somites.

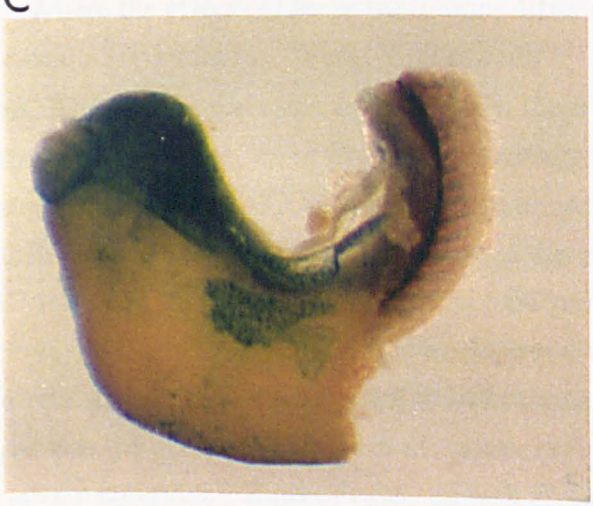
B. Embryo co-injected with nuc.β-gal RNA and XrelAΔSP RNA (200 pg/embryo).
Note - This embryo clearly has XrelAΔSP RNA in the somites as shown by the regular chevron pattern of X-gal staining (pale blue) in the somites.

C. Embryo co-injected with nuc.β-gal RNA and XrelAΔSP RNA (1ng per embryo). Preservation of these embryos was poor, but in this case the fracturing fortuitously reveals X-gal staining in the somites.

A

B

C



6.3. Conclusions and Discussion.

6.3.1. There are only limited similarities between the XrelAΔSP and XFD phenotypes

Expression of XrelAΔSP in embryos by injection of mRNA at the two cell stage results in embryos with a reproducible range of defects. Externally these embryos have a number of similarities to the phenotype caused by expression of the dominant negative FGF receptor XFD. These similarities may have their origins in the eccentric mode of gastrulation, which occurs in both XrelAΔSP and XFD expressing embryos. This involves a failure, or delay, in closure of the blastopore during gastrulation (in some embryos closure was not observed until the late neurula stages), followed by 'migration' of the dorsal mesoderm around one side of the exposed yolk plug. Various potential explanations of this behaviour are discussed later (section 8.1.1.). At later stages, the outcome of this failure to close the blastopore is a large open yolk plug behind the head. Another similarity between the two phenotypes is an inhibition of tail extension. A number of recent reports support a model of tail extension as a continuation of gastrulation. For example, expression of Xbra and Xnot.2 can be followed from the blastopore lip to distinct cell populations in the tailbud (Smith *et al.*, 1991; Gont *et al.*, 1993). This region has also been reported to retain potent trunk organising activity until the late stages of development (Gont *et al.*, 1993). The gastrulation and tail extension defects seen in both XFD and XrelAΔSP embryos could therefore result from inhibition of the same set of developmental/molecular processes.

The similarities between the XFD and XrelAΔSP phenotypes appear to be largely 'mechanical'. Where the effects of these two clones differ significantly is in the ability to inhibit tissue differentiation. While XFD can inhibit the differentiation of notochord and somites, XrelAΔSP expressing embryos form these tissues normally, at least on one side of the open yolk plug, even from cells receiving the highest dose of injected mRNA. Histological analysis of XFD expressing embryos has revealed that in extreme cases no differentiated neural tissue is apparent in the trunk (C. Beck, pers. comm.) In XrelAΔSP embryos, on the other hand, neural tube was always seen along the length of the embryo, although it generally failed to close and was smaller than in controls. The only (obvious) complete loss of a major differentiated tissue in XrelAΔSP expressing embryos was the occasional absence of one or both eyes, seen more consistently in more severe embryos. Loss of eyes, as well as, more severe head

7. Analysis of phenotypes - Marker expression

7.1. Introduction

One way of testing the FGF inhibition hypothesis is to study the expression of various markers whose response to XFD expression has been previously characterised. In the event of these experiments failing to uphold the hypothesis they may provide a starting point for other models of the origins of the XrelAΔSP phenotype. Of course, it is possible to test for inhibition of FGF signalling more directly using animal cap assays. Unfortunately, this came under the remit of other work being carried out in the lab to study the effects of rel dominant negatives on mesoderm induction in general over a wide range markers .

A range of probes was chosen, subject to their availability, based on the FGF inhibition hypothesis, and more generally on direct relevance to the phenotype. In order to put the data from these experiments into context I have briefly summarised what is known about each of the markers used including expression patterns and possible developmental role (if models exist).

7.1.1. *Brachyury*

As discussed in the introduction *Brachyury* is a gene intimately associated with mesoderm formation in vertebrates. The *Xenopus Brachyury* gene (*Xbra*) is expressed throughout the prospective mesoderm in the blastula (its expression is an immediate early response to mesoderm induction), but during gastrulation it becomes localised in the notochord, and later in the tailbud (Smith *et al.*, 1991). *Xbra* is expressed in animal caps in response to induction by both activin (as an immediate early response) and bFGF (Smith *et al.*, 1991; LaBonne *et al.*, 1995). Expression of *Xbra* in embryos is dependent on signals inhibited by dominant negative activin and FGF receptors (Hemmati-Brivanlou and Melton, 1992; Amaya *et al.*, 1991). Xbra itself has ventral and lateral type mesoderm inducing activity when overexpressed in animal caps (Cunliffe and Smith, 1992; Orielly *et al.*, 1995)

Unlike most of the other markers which were isolated because of homology to developmentally important genes in *Drosophila*, or in functional screens, the phenotype associated with a null allele of *Brachyury* in mice was known long before the gene was cloned (Chesley, 1935). More recently a mutation in zebrafish called *no*

tail has been shown to be due to a mutation in the zebrafish *Brachyury* gene. Zebrafish *no tail* (*Brachyury* null) embryos lack notochord completely. This is due to failure of its formation during gastrulation, rather than later degeneration. They also exhibit deformed somites (although segmentation does take place and muscle fibres differentiate, the myotome is block-like rather than chevron shaped). Wild-type cell transplantation studies show that this is due to the effects of Brachyury on notochord differentiation, rather than a direct effect on muscle differentiation (Halpern *et al.*, 1993). Halpern *et al.* interpret this as implying that *Brachyury* is necessary for a subsequent signalling event which occurs between notochord and somitic mesoderm. It should be noted that this signal does not appear to be the same as the one secreted by notochord required for neural floor-plate differentiation as this structure develops normally in *no tail* embryos (probably due to the presence of undifferentiated notochord precursor cells which secrete it). As one would expect from the name, tail formation is inhibited in these embryos. This fits well with a model of tail formation as a continuation of gastrulation, which would seem to make intuitive sense, as a similar arrangement of dorsal mesodermal structures is formed in the trunk during gastrulation. In fact, expression of *Xbra* and a second dorsal mesodermal marker, *Xnot2*, can be followed from the blastopore lip to distinct cell populations in the developing tailbud, which interestingly retains potent organiser activity until late stages of development (Gont *et al.*, 1993).

Increasing evidence suggests that the product of the *Brachyury* gene is a DNA binding protein. A consensus binding site has been determined using a PCR selection assay starting from a random pool of oligonucleotides. (Kispert and Hermann, 1993)

7.1.2. Xwnt-8

Xwnt-8 is a member of the wnt family of signalling proteins related to the oncogene *int1* and the *Drosophila* gene *wingless* (*wg*) (Christian *et al.*, 1991). The precise expression pattern of Xwnt-8 appears to be the subject of some dispute. According to Smith and Harland (1991), expression begins at stage 9/10 in the whole mesoderm, excluding the organiser. During gastrulation the expression becomes progressively more ventrally restricted. By the early neurula it is expression is restricted to posterior ventral mesoderm. At stage 16 a low level of expression remains in the ventral posterior mesoderm and is also seen in two patches lateral to the anterior neural plate. Christian and Moon have also detected expression in lateral plate mesoderm at the neurula stage (Christian and Moon, 1993). This expression

data was obtained from whole mount *in situ* hybridisation experiments using a digoxigenin labelled riboprobe which, due to penetration problems, tends not to detect vegetal expression. By hybridising using digoxigenin labelled probes to sections Lemaire and Gurdon have detected vegetal expression at stage 10. By staining adjacent sections for expression of *Xwnt-8* and *Xbra* they showed that dorso-laterally the expression of *Xwnt-8* is almost entirely vegetal whereas ventrally it is also present equatorially.

The possible developmental function of *Xwnt-8* and its activities are discussed in detail in the introduction (see page 48).

Xwnt-8 expression in animal caps can be induced by both FGF and activin. Like *gsc* expression *Xwnt-8* expression induced by activin in animal caps is not inhibited by XFD (LaBonne and Whitman, 1994).

7.1.3. *goosecoid*

goosecoid (*gsc*) is a homeobox containing gene related to the *Drosophila* homeobox genes *gooseberry* and *bicoid* (Blumberg *et al.*, 1991). Its expression is restricted to the organiser at stage 10, and during gastrulation becomes restricted to the prospective pre-chordal plate (Cho *et al.*, 1991). *gsc* expression is an immediate early response to mesoderm induction by activin in animal caps (Cho *et al.*, 1991), although relatively high levels of activin are required compared to those needed to induce *Xwnt-8* or *Xbra* expression. Expression is not induced by bFGF, and is not inhibited by XFD expression, either in whole embryos, or in animal caps treated with activin (Amaya *et al.*, 1993; LaBonne and Whitman, 1994)

Gsc has the ability to induce secondary trunk formation when injected ventrally during early cleavage, and can do so in a non-cell autonomous manner (Cho *et al.*, 1992; Niehrs *et al.*, 1993), suggesting that *gsc* can function to induce the expression of (trunk) organiser signals. *Gsc* expression can also effect cell migration during gastrulation so that overexpression in dorsal blastomeres causes expressing cells to migrate anteriorly and contribute to the pre-chordal plate (Niehrs *et al.*, 1993)

7.1.4. Xnot

Xnot is a homeobox gene which is most closely related (in its homeobox domain) to the *Drosophila* gene *empty spiracles* (Von Dassow, *et al.*, 1993). It is expressed at a low level maternally, and initial zygotic transcription is ubiquitous (at stage 9). By stage 10.5 transcripts have become restricted to the organiser, but in non-involuted tissue at some distance from the lip which may be prospective dorsal trunk mesoderm (trunk organiser), and also to a thin belt of cells also some distance from the lip, which von Dassow and colleagues speculate may mark the limit of mesoderm involution. This gradual restriction of expression is dependent on translation, as cycloheximide treated embryos continue to express *Xnot* ubiquitously at control stage 12. Later, in the gastrula and neurula stages, expression is limited to the dorsal midline in all three germ layers (i.e.:- in the archenteron roof, notochord, and the floorplate of the neural tube.)

Xnot expression can be induced in animal caps by both FGF and activin. In whole embryos expression of *Xnot* in the marginal zone can be induced by *Xwnt-8*, and is inhibited by BMP-4, and by expression of a dominant negative FGF receptor (VonDassow *et al.*, 1993).

7.1.5. Pintallavis

Pintallavis was originally isolated in a PCR screen for members of the winged helix family of transcription factors which includes rat hepatocyte nuclear factor 3 (HNF-3) and *Drosophila forkhead* (Ruiz-i-Altaba and Jessel, 1992). It is expressed initially throughout the mesoderm late blastula, but becomes rapidly localised to the organiser region (by stage 10½). By stage 12 *pintallavis* is expressed in all three germ layers in the dorsal midline, and at lower levels in the pre-chordal plate (mesoderm only). Expression in the midline of the neural plate is restricted to deep layer cells. As expression in the ectoderm is not seen in complete exogastrulae it seems likely that this expression requires induction by the mesoderm which comes to underlie the ectoderm during gastrulation.

There is currently no published data on what signals can induce the expression of *pintallavis* in animal caps, or on the effects of dominant negative receptors on its expression in whole embryos.

Two sets of experiments have given some indication of the developmental roles of *pintallavis*. Expression in animal caps does not induce mesoderm formation, however co-expression with *Xbra* in animal caps leads to the formation of notochord, suggesting that *pintallavis* may be downstream of notochord inducing signals in the organiser (Orielly *et al.*, 1995). However, as overexpression is not sufficient to induce a second axis, or to produce radially dorsalised embryos it is obviously not capable by itself of inducing organiser signals. In fact, overexpression of *pintallavis* by bilateral injection of RNA at the two cell stage produces shortened embryos lacking (or having severely reduced) anterior neural structures, but exhibiting an expansion of posterior neural structures (Ruiz-i-Altaba and Jessel, 1992). These effects can be seen at neurula stages as a lack of formation of normal anterior neural folds. This has been taken to imply a role in specification of posterior neural fate, which would fit with its neural expression pattern, and the fact that this expression appears to be dependent on (vertical) neural inductive signals.

7.2. Results

In situ hybridisations were carried out to embryos injected with of XrelAΔSP RNA, as described in section 2.3.9., using antisense probe RNA probes transcribed from the appropriate vector (see Appendix 2) Unless otherwise stated, injections were carried out bilaterally at the two cell stage with approximately 1 ng (2 x 500 pg injections) of RNA per embryo. Where albino embryos were used they were lightly stained with Nile blue to make staging easier and to identify embryos showing signs of necrosis so that they could be discarded. As a control for non-specific staining, uninjected embryos were taken through the *in situ* process without the addition of probe. In some cases non-specific staining was seen in the blastocoel and also sometimes in the archenteron (data not shown). Each figure includes descriptive scoring of all stained embryos and pictures of representative examples, where appropriate.

7.2.1. *Xbra*

Embryos bilaterally injected with 1 ng of XrelAΔSP which have been fixed and stained for *Xbra* expression at stage 10 showed the loss of arcs of staining cells, as illustrated in figure 38. Loss of some expression could be detected at injection concentrations down to 200 pg per embryo (see figure 38). Complete loss of expression was rarely observed.

Xbra in situ hybridisations were also carried out with embryos injected with p50ΔSP RNA, and XrelAΔSP222 mRNA both at 1ng of mRNA per embryo. No inhibition of *Xbra* expression was seen in these embryos (data not shown).

Unfortunately, because of sensitivity problems it was not possible to carry out equivalent experiments in late blastulae in order to see if immediate early expression of *Xbra* was also affected.

7.2.2. Unlike XFD there is no dorsal-ventral difference in the sensitivity of *Xbra* expression to XrelAΔSP.

The expression of *Xbra* and another pan mesodermal marker, *Xcad*, is more sensitive to inhibition by XFD dorsally than ventrally (Northrop and Kimelman, 1994). This can not be accounted for by dorsal-ventral differences in expression of FGFR-1. The difference in sensitivity may instead result from differences in the

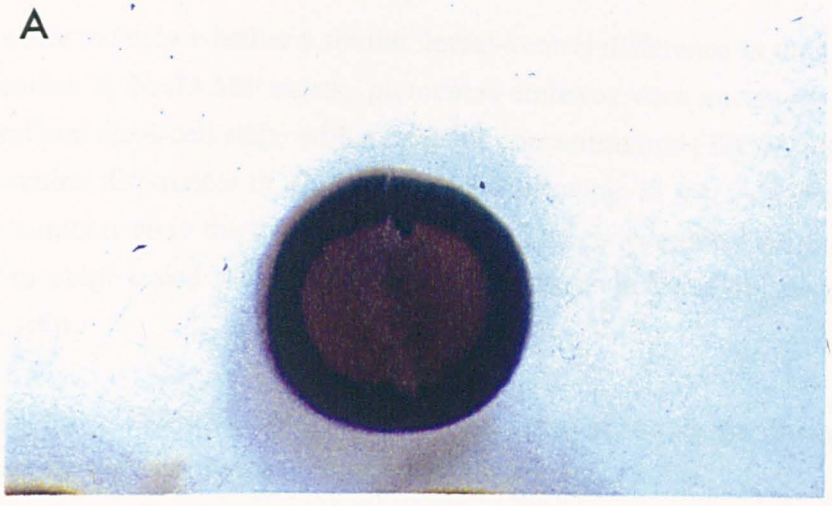
FIG. 38:- XrelAΔSP inhibition of *Xbra* expression at stage 10

Albino embryos bilaterally injected with approximately 1ng/embryo of XrelA ΔSP RNA at the two cell stage were fixed at stage 10 and stained for the expression of *Xbra* by whole mount *in situ* hybridisation.

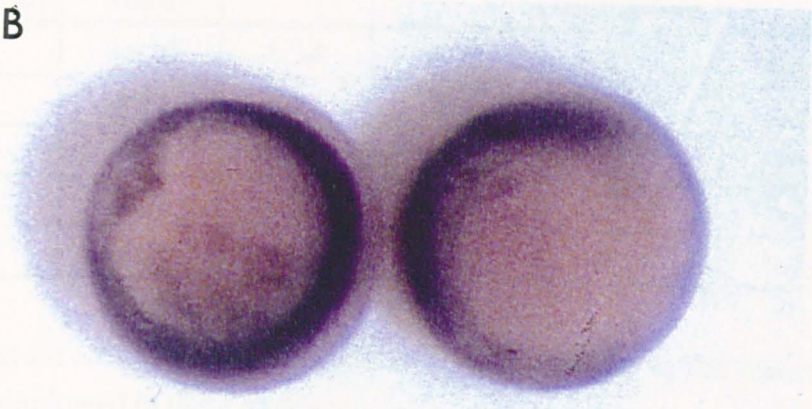
A. Control (vegetal view), showing strong expression in a ring around the equator

B. Injected (vegetal view) - showing arcs of lost expression.

A



B



strength of the FGF signal, either due to a variation in the actual concentration of secreted FGF or variation in affinity as regulated by proteoglycans. Alternatively the difference could be due to the presence of additional factors which can compensate to some degree for a diminution, but not an elimination, of the FGF signal.

In order to study whether a similar dorsal-ventral difference in the inhibition of *Xbra* expression by XrelAΔSP exists, pigmented embryos were injected quadrilaterally at the 4-cell stage with a range of concentrations of RNA and scored for dorsal-ventral differences in *Xbra* expression following *in situ* hybridisation. The results are summarised in the table in figure 39. At no concentration of injected XrelAΔSP message tested were dorsal-ventral differences in the inhibition of Xbra expression seen.

FIG. 39:- Table showing results of experiment to test for dorsal-ventral differences in response to XrelAΔSP expression.

		Stain Lost:-			
RNA Injected (ng/injection)	**Complete ring of stain**	**Dorsally**	**Ventrally**	**Laterally only, or ambiguous**	**No Stain**
-	30/30	0/30	0/30	0/30	0/30
1	0/24	8/24	7/24	5/24	4/24
0.5	4/29	9/29	10/29	6/29	0/29
0.2	20/23	0/23	2^R/23	1^R/23	0/29
0.1	26/26	0/26	0/26	0/26	0/26

n^R The affected arc of staining was reduced (i.e.:-still faintly visible) rather than completely eliminated in these embryos.

7.2.3. Pintallavis

Embryos injected with XrelAΔSP (1ng/embryo) frequently show loss of most or all of the dorsal arc of expression of *pintallavis* at stage 10.5 (see figure 40). At stage 12 injected embryos fell into three groups with respect to *pintallavis* expression. In some embryos expression was completely eliminated (data not shown). Where expressing cells remained they frequently failed to involute (see figure 41 b) showing that lack of involution of dorsal cells does not correlate with loss of dorsal expression

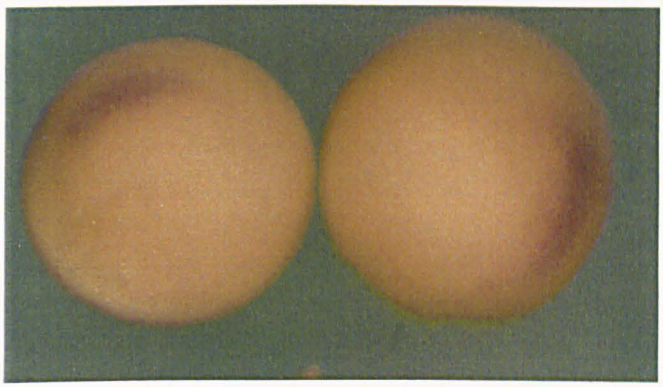
FIG. 40:- *Pintallavis* at stage 10½

Albino embryos bilaterally injected with approximately 1ng/embryo of XrelAΔ SP RNA at the two cell stage were fixed at stage 10½ and stained for the expression of *pintallavis* by whole mount *in situ* hybridisation.

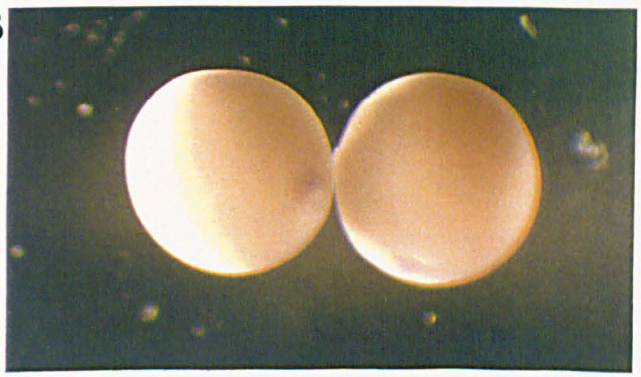
Scoring:- 4/12 normal, 1/12 normal except staining appears to be on surface 5/12 reduced to spot, 2/12 staining completely eliminated.

The figure shows a control embryo (**A**) and two XrelAΔSP injected embryos (**B**), one lacking any *pintallavis* expression (right) and one with a small remaining spot of expressing cells. (**C**) is a comparison of a cleared control (left) and a cleared a XrelAΔSP expressing embryo. The control is seen looking down on the vegetal pole and has a broad dorsal arc of expressing cells. The injected embryo (right), seen in a dorsal view, has a much reduced dorsal arc of expression.
Note - the blastocoel is outlined in these embryos by the non-specific precipitation of stain. This is phenomenon is also frequently seen in negative control embryos taken through the *in situ* hybridisation procedure in the absence of probe (data not shown).

A



B



C

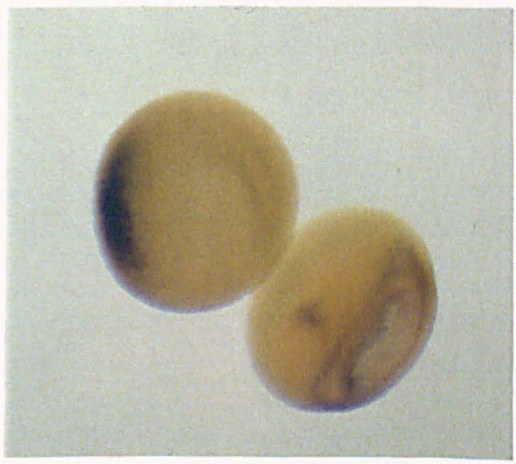


FIG. 41:- Pintallavis at stage 12

Albino embryos injected bilaterally with approximately 1ng/embryo of XrelAΔ SP RNA at the two cell stage were fixed at control stage 12 and assayed for expression of *pintallavis* by *in situ* hybridisation.

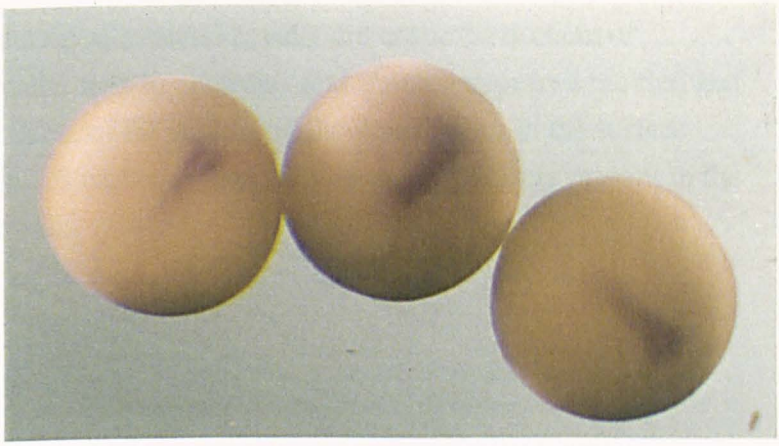
Scoring:- 3/13 No visible expression with open yolk plugs, 6/13 small disorganised clumps of non-involuted cells on one side of open yolk plug, 4/13 Involution of expressing cells has occurred and yolk plug almost closed but expression seems to be broader and closer to the surface than in controls.

A. Controls showing expression in the dorsal midline. The domain of expression is broader posteriorly and tails off anteriorly.

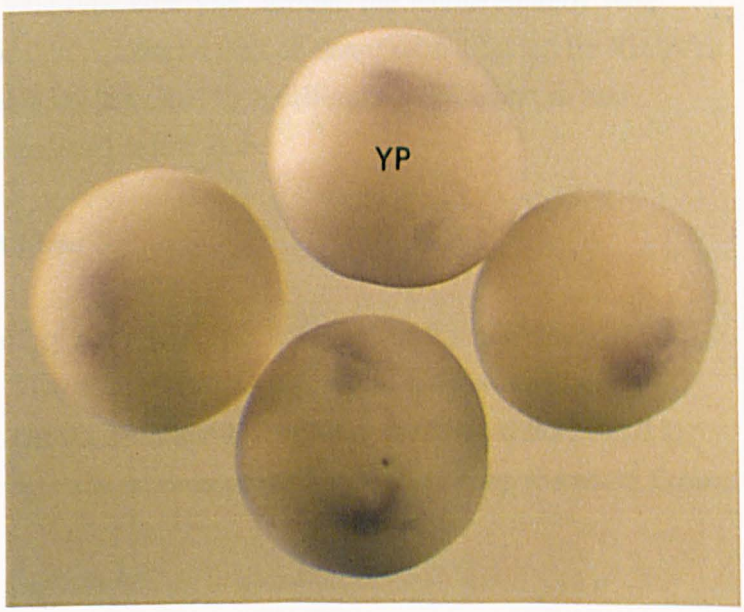
B. Injected embryos showing clumps of non-involuted expressing cells on one side (presumably dorsal, although it is not possible to confirm this) of their exposed yolk plugs (YP).
NB:- The bottom embryo has a second dark patch. This is a forcep mark, not stain.

C. Injected embryos showing involution of expressing cells. However, expression appears broader than in controls, and unlike controls some staining appears to be on the surface (staining in the midline of the neural plate at this stage is normally restricted to deep layer cells (Ruiz-i-Altaba and Jessel, 1992).

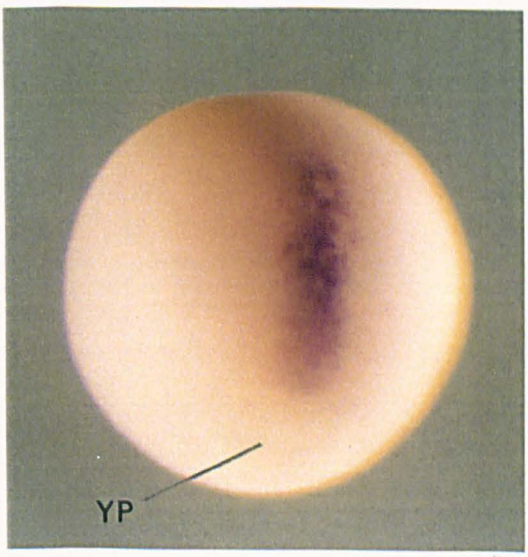
A



B



C



of *pintallavis*. When involution of expressing cells did occur the domain of expression was laterally broader than in controls, and staining appeared mottled and stronger, possibly because some of the staining cells were present in the surface ectoderm (figure 41C). This is unlike controls where staining is only present in the deeper layer of neural plate cells.

7.2.4. Xnot

Embryos injected with 2 ng of XrelAΔSP RNA frequently lose the majority of their dorsal expression, leaving a thin ring of equatorial expression of the type seen both laterally and ventrally in wild type embryos, around the whole equator (see figure 42). In no case was this thin equatorial ring of expression affected by XrelA Δ SP expression, suggesting that it is controlled by a different mechanism to that controlling the dorsal (trunk organiser) expression.

7.2.5. *Goosecoid* and *Xwnt-8*

Injection of 1 ng per embryo of XrelAΔSP is sufficient for inhibition of both *Xwnt-8* and Gsc expression. However, whereas *Gsc* expression was either completely inhibited (7/11) as shown in figure 43, or completely unaffected (4/11), *Xwnt-8* expression was only ever reduced over a small arc (10/13), as shown in figure 44.

FIG. 42:- Xnot at stage 10½

Albino embryos bilaterally injected with approximately 1ng/embryo of XrelAΔSP RNA at the two cell stage were fixed at control stage 10½ and stained for the expression of *Xnot* by whole mount *in situ* hybridisation.

A dorsal arc of strongly staining cells can be seen in the control (left), along with a thin ring of faintly staining cells around the rest of the equator (ventral and lateral). In the injected embryos (right) the broad dorsal arc of strongly staining cells is lost leaving a thin ring of low level expression, as seen ventral and lateral mesodermal cells in the control, extending around the circumference of the embryo.

Scoring:- 5/14 unaffected, 9/14 reduction of dorsal staining to lateral/equatorial levels (as in photo).

FIG. 43:- Gsc expression is inhibited by XrelAΔSP.

Albino embryos bilaterally injected with approximately 1ng/embryo of XrelASP RNA at the two cell stage were fixed at stage 10 and stained for the expression of *Gsc* by whole mount *in situ* hybridisation.

A dorsal arc of expressing cells (corresponding to the organiser) can be seen in the control (left) but is lost in the injected embryo (right).

Scoring:- Complete inhibition of expression in 7/11 embryos; no inhibition of expression in the remaining 4

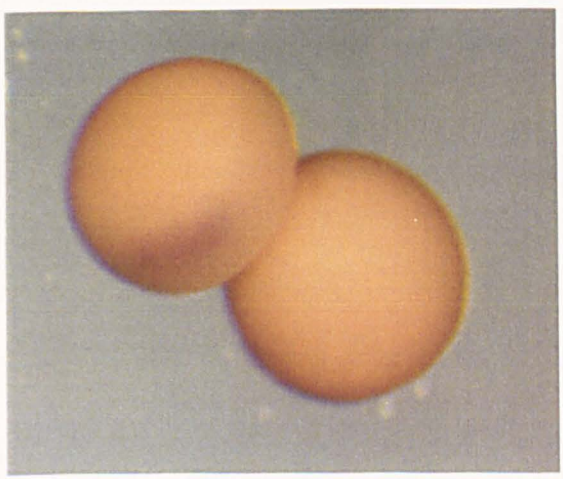


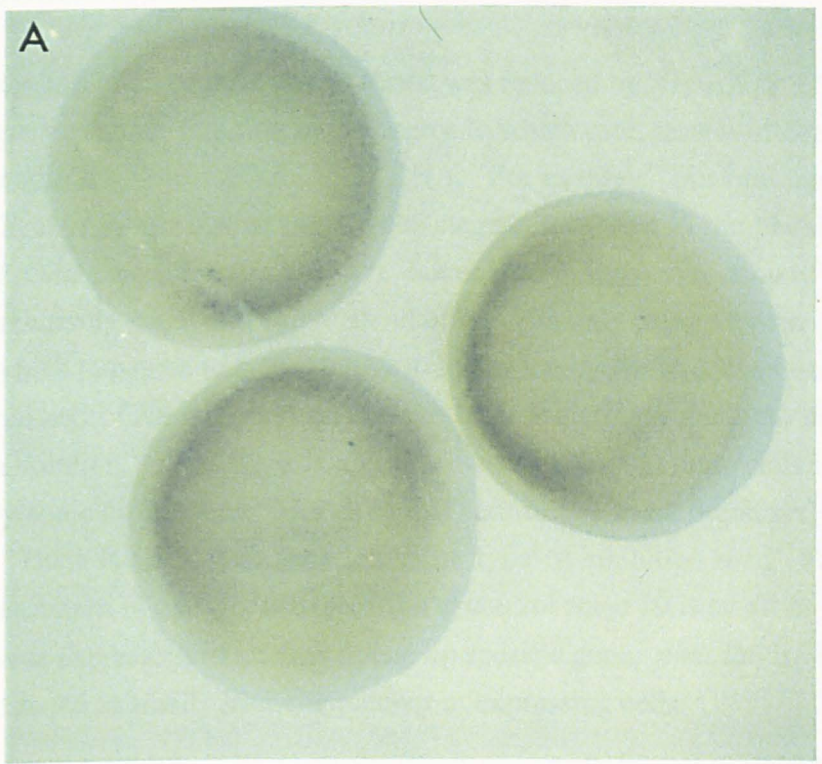
FIG. 44:- Xwnt-8 expression is inhibited by XrelAΔSP

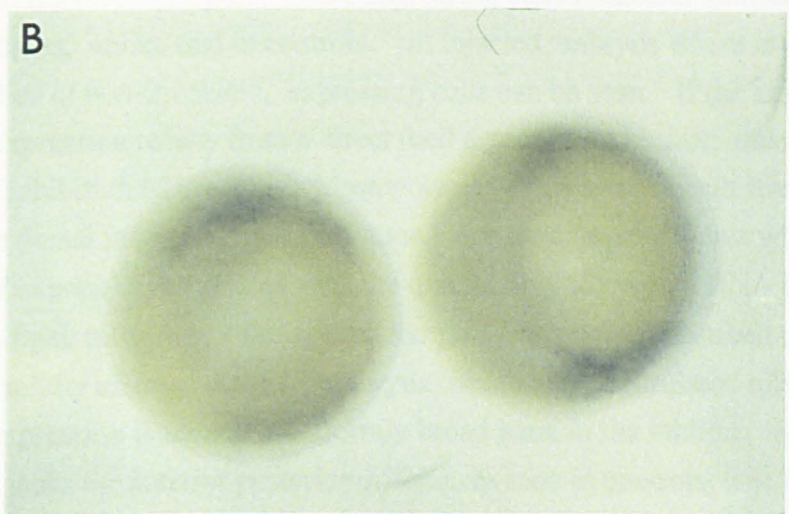
Albino embryos bilaterally injected with approximately 1 ng/embryo of XrelASP RNA at the two cell stage were fixed at stage 10½ and stained for the expression of *Xwnt-8* by whole mount *in situ* hybridisation. All the embryos shown were cleared before photography.

Scoring:-
Reduced expression over a small arc in 10/13, the rest were unaffected. Interestingly this arc of reduced expression was always ventral (rather than lateral), and lateral expression appeared to be stronger than in controls. However, too few embryos were tested in this experiment to be able to draw definite conclusions from this.

A. A vegetal view of controls showing the ventral and lateral arc of expression in the mesoderm.

B. Injected embryos, both with a small arc of reduced expression on the ventral side.


A


B

7.3. Conclusions and Discussion

Expression of all of the markers tested was reduced by XrelAΔSP expression, although there was some variation in the degree to which each gene is effected by the same concentration of injected XrelAΔSP RNA. For example, bilateral injection of 1 ng injected XrelAΔSP mRNA at the two cell stage is sufficient to completely inhibit large arcs of Xbra expression (up to 180°), but only to reduce expression of Xwnt-8, and then in relatively small arcs (up to about 60°). The only example seen of expression which appeared to be refractory to inhibition by XrelAΔSP expression is a thin equatorial belt of Xnot expression at stage 10.5, which may mark the limit of mesoderm involution. This suggests that expression of Xnot in these cells is controlled by some different mechanism to that controlling 'trunk organiser' expression. There is also subtle variation in the 'type' of inhibition seen. For example, inhibition of *goosecoid* expression at control stage 10 is an all or nothing effect, whereas expression of another dorsal lip specific gene, *pintallavis*, is frequently reduced to small, irregular patches of expressing cells.

Although *pintallavis* expression at control stage 12 was inhibited in a proportion of injected embryos, where expressing cells remained their distribution was, in all cases, unlike that in controls. In injected embryos where involution has failed, patches of non-involuted, expressing cells can be seen. If the ability to inhibit pintallavis expression results from a direct (cell autonomous) effect, then this result implies that inhibition of involution/blastopore closure may not result from a direct effect on the dorsal involuting marginal zone. It would be easy to test whether inhibition of expression correlated with the distribution of injected RNA by using co-injected nuc.βgal. mRNA as a lineage marker (see experiment described on page 125). In the more mildly affected stage 12 embryos which have gastrulated relatively normally, expression is seen in a uniformly broad band in the midline, which is both broader and lacks the anterior posterior differences seen in controls. (see figure 41C). Lateral broadening of expression may be a reflection of a reduction in convergence and extension in the dorsal midline cells, i.e. expression may be in the same cells, but these cells are more spread out laterally.

XrelAΔSP and XFD differ in their effects on mesodermal marker expression. For example, although they both inhibit Xbra expression, Xbra expression does not exhibit dorsal-ventral differences in sensitivity to XrelAΔSP, as it does to XFD. If this difference in sensitivity to XFD expression is due to differences in the levels of active FGF (and therefore in the strength of the FGF signal), then one would expect an

inhibitor of FGF signalling to show the same dorsal ventral differences in its effects. Another illustration of the difference is seen in their effects on *goosecoid* expression. The inhibition of *gsc* expression by XrelAΔSP shows that the phenotype cannot be due to an inhibition of FGF signalling alone, as this marker is unaffected by expression of XFD. It is unclear whether the lack of the equatorial domain of Xnot expression represents a difference with XFD, as the only mention of XFD inhibition on Xnot expression in the literature (vonDassow *et al.*, 1993) involved scoring at control stage 12, after this domain of expression is lost.

It is difficult to reconcile this relatively broad inhibition of marker expression with the relatively mild phenotype (no tissue type is absent) characterised in the previous chapter. Inhibition of *Xbra* expression dorsally, for example, would be expected to inhibit notochord development (as in the null phenotype in zebrafish). One possible solution to this apparent paradox could be that inhibition of expression of say, *Xbra*, is confined to a narrow window. Future work could investigate this possibility further.

If some markers are only inhibited at earlier stages, this is unlikely to be due to instability of the message. Experiments carried out to test for the persistence of XrelA ΔSP RNA in injected embryos by *in situ* hybridisation showed that RNA was still present at high levels (seen after seconds in the staining reaction instead of the hours (at least) required to detect even abundant endogenous RNA) in the late neurula (data not shown).

8. Discussion

8.1. Discussion of dominant negative phenotypes.

8.1.1. Discussion of the developmental mechanism of action of XrelAΔSP.

As discussed in chapter 6, one similarity between XFD and XrelAΔSP injected embryos is a failure, or delay, in closing the blastopore during gastrulation, followed by 'migration' of the dorsal mesoderm around one side of the exposed yolk plug. The origins of this behaviour in XFD and XrelAΔSP expressing embryos may, or may not be the same, but for convenience I shall deal with various possible explanations of this behaviour together. Embryos lacking an animal cap and non-involuting marginal zone (NIMZ) can succeed in closing the blastopore (Keller and Janser, 1992). Failure to close the blastopore in these embryos could therefore be a result of a direct effect on cell behaviour in the involuting marginal zone (IMZ). However, it also seems plausible to suggest that inhibition of epiboly in the animal cap could physically restrain the IMZ cells from performing this task. A number of explanations could be proposed for the behaviour of the dorsal mesoderm during gastrulation in these embryos. As these embryos frequently exhibit retention of the blastocoel, one possibility is that it is an indirect effect of an inhibition of blastocoel collapse (although this could equally be a result, as a cause). There is some evidence that blasotceol collapse is an active, rather than a passive process, based on the result that overexpression of certain G-protein α-subunits inhibit blastocoel collapse (Otte, 1992). Interestingly these embryos eventually develop normally, apart from a 'ventral blister' due to the retained blastocoel, suggesting that inhibition of blastocoel collapse does not inevitably lead to gastrulation around the yolk plug. Another possible explanation which has been proposed is that the entire dorsal mesoderm in these embryos behaves like the prospective head mesoderm in wild-type embryos, i.e.- it actively migrates, but does not undergo medio-lateral intercalation and so does not converge and extend (Isaacs *et al.*, 1994). Ray Keller has proposed a model of gastrulation which suggests that co-ordinated patterns of mediolateral interacalation behaviour (MIB) of IMZ cells provide the driving force for both blastopore closure and convergence and extension of the dorsal mesoderm during gastrulation (Shih and Keller, 1992b; Keller *et al.*, 1992). Inhibition of mediolateral intercalation behaviour may therefore be an explanation for inhibition of blastopore closure and for the eccentric gastrulation of XFD expressing embryos. The one piece of data which seems to argue against this model in the case of XrelAΔSP embryos is the expression

of pintallavis in these embryos at control stage 12. As discussed in section 7.3., although expression is inhibited in a proportion of injected embryos, where expression remains it is frequently seen in patches of non-involuted cells. If the inhibition of expression is a direct effect then it would be problematic to attribute inhibition of involution to a direct effect on these cells as well.

The control of the co-ordinated patterns of cell behaviour which drive gastrulation, and the induction of mesoderm may actually involve the same inducing factors. Certainly, no gastrulation takes place in embryos where mesoderm induction has been blocked by expression of the dominant negative activin receptor Δ1XAR1. The ability of mesoderm inducing factors to induce animal caps, and individual animal cap cells, to display the various morphogenic behaviours associated with IMZ cells during gastrulation has been extensively characterised (Howard and Smith, 1993). Activin can induce massive convergent extension in animal caps, and a variety of behaviours in single cells including elongation and spreading on fibronectin. FGF alone is able to induce only a subset of these behaviours in single cells, and only induces a small amount of convergent extension in whole caps. However, FGF signalling is necessary for induction of convergent extension in animal caps by activin, as shown by the inhibition of this behaviour by XFD (Cornell and Kimelman, 1994).. XrelAΔSP also has the ability to inhibit activin induced convergent extension of animal caps (C.Beck, Personal communication). This supports the suggestion that the gastrulation defects in both XFD and XrelAΔSP expressing embryos are due to a direct effect on the behaviour of IMZ cells.

8.1.2. Discussion of the molecular mechanism of action of XrelAΔSP.

<u>The evidence for and against squelching by XrelAΔSP.</u>

The failure of XrelAΔSP to inhibit transactivation from the thymidine kinase promoter, at least to the degree that wild-type XrelA does, is good evidence that transcriptional squelching by XrelAΔSP does not occur. However, the degree of experimental error in this experiment means that a lower level of squelching by XrelA ΔSP than by wild-type protein cannot be ruled out. This result may seem suprising in light of the fact that XrelAΔSP contains the complete transactivation domain and nuclear localisation signal of XrelA (presumably transcriptional squelching takes place in the nucleus). However, as discussed in section 5.4.3., the squelching activity of a number of other transactivating factors has been shown to require the presence of a functional DNA binding domain, so this result is not without precedent. The apparent

lack of squelching by XrelAΔSP suggests that, although the transactivation domain is also required for the phenotypic effects of XrelAΔSP expression, these effects are due to some interaction which requires this domain, other than with some general transactivating factor.

The inhibition of expression (albeit to varying degrees), of all the markers studied, might seem to support the squelching hypothesis, but it would probably be rash to draw such a conclusion on the basis of the analysis of such a limited range of markers. Other work carried out with XrelAΔSP into its effect on mesoderm induction in caps also suggests that its effects are more specific than a general squelching of transcription (see below).

One of the more compelling arguments against the squelching hypothesis is that the phenotype caused by injection of XrelAΔSP mRNA into embryos is less severe than might be expected for a general inhibitor of transcription. It is certainly less severe than that caused by overexpression of XrelA (at least at high levels), which is probably attributable to squelching.

What are the potential candidates for interaction with XrelAΔSP?

When the dominant negatives used in this work were designed the only known interactions of NF-κB subunits p50 and p65 were with other rel family members. Since then the interaction of p65 and p50 with a number of different transcription factors has been characterised. These transcription factors are therefore candidates for the factor(s) titrated by XrelAΔSP in *Xenopus* embryos.

Both p65 and p50 interact with a number of bZIP type DNA binding proteins (see table - figure 45), characterised by the presence of a leucine zipper structure adjacent to a basic DNA binding region. One group of bZIP proteins which have been shown to physically interact with p65 and p50[20] are the CCAAT/enhancer binding protein (C/EBP) family members C/EBPα, CEBPβ (previously known as NF-IL6), C/EBPγ (previously called Ig/EBP-1) and C/EBPδ (Stein *et al.*, 1993). Interactions with the C/EBP family, which can occur in the absence of DNA are mediated through the rel homology domain, so may also be found for other rel family members, and appear to require dimerisation, as the dimerisation deficient relA mutant p65Δ is

[20] p50 has only been shown to interact with C/EBPβ.

unable to interact. Functionally this interaction appears to serve to inhibit binding to κB sites and potentiate binding of the resulting complex to C/EBP binding sites (for which $(p65)_2$ and $(p50)_2$ NF-κB alone have no affinity). The ability to inhibit binding to NF-κB sites is of obvious interest given the failure of attempts to detect κB binding activity attributable to endogenous XrelA.

Another family of bZIP containing proteins which has members capable of interacting with NF-κB subunits is the ATF/CREB family. Physical interactions with NF-κB subunits in the absence of DNA and which also appear to involve the RHD, have been detected with ATFa, ATF2 and ATF3 (p50 only), and c-jun (Kaszubska *et al.*, 1993). Unlike the interactions with the C/EBP family, these interactions seem to function to allow co-operative binding to promoters containing adjacent ATF and κB binding sites.

NF-κB has also been shown to bind co-operatively with the zinc finger containing transcription factor Sp1, to the HIV-LTR, which contains 2 κB sites adjacent to three Sp1 sites. This interaction has not been shown to occur in the absence of DNA (Perkins *et al.*, 1993 and 1994). This interaction also involves the RHD. Other possible candidates for titration by XrelAΔSP are HLH box proteins, for which circumstantial evidence of an interaction exists (see introduction, page 27), and a DSp1 homologue (again for which there is circumstantial evidence in vertebrates (see introduction page 26).

The range of likely targets for XrelAΔSP is somewhat reduced by the finding that the C-terminal domain is necessary for XrelAΔSP to have its phenotypic effects. As discussed in the introduction the c-terminal domain is highly divergent from relA, so it is difficult to see how any interactions might be conserved.

FIG. 45:- Table of interactions between bZIP proteins and NF-κB subunits.

bZIP protein	p50	p65
C/EBPα	Unknown	+
C/EBPβ	+	+
C/EBPγ	Unknown	+
C/EBPδ	Unknown	+
ATFa	+	+
ATF2	+	+
ATF3	+	No Interaction
c-Jun	+	+

Discussion of the possible effects of XelAΔSP expression on FGF signaling.

From the data presented in Chapters 6 and 7, it is clear that, despite the apparent external similarities, XrelAΔSP and XFD do not have identical phenotypic effects. The main similarities are in the method of gastrulation, the shortening of the trunk, and the inhibition of tail extension. One potential model suggested by these similarities is that XrelAΔSP somehow inhibits the morphogenic effects of FGF, but not its ability to induce/maintain the formation of mesoderm. However, the inhibition of expression of the early mesodermal markers by XrelAΔSP, some of which at least are thought to have a role in the specification of tissue type, suggests that this is not the case. The range of markers whose expression is inhibited by XrelA ΔSP is, if anything, broader than that inhibited by XFD (at least at the early gastrula stages). For example, *goosecoid* expression is inhibited by XrelAΔSP, but not by XFD (according to the literature at least).

Work on the effects of XrelAΔSP on induction of mesodermal markers in caps has shown that XrelAΔSP is able to completely inhibit the induction of all mesodermal markers in animal caps by FGF, but not by BMP4, or by activin, although the induction of some markers in activin treated caps is reduced compared to controls (C.Beck, personal communication). This result is odd in that it is not consistent with the effects of XFD, which is able to abolish induction of a number of markers in animal caps by activin (LaBonne and Whitman, 1994; Cornell and Kimelman, 1994). Various potential models which explain this are currently being investigated, but these fall beyond the scope of this thesis. While this data supports the idea that

the effects of XrelAΔSP are very specific, and that XrelAΔSP is an efficient inhibitor of mesoderm induction by FGF, it is currently not easy to construct a comprehensive model which accounts for both the phenotype in whole embryos and the data from animal cap experiments.

8.2. The function and control of endogenous XrelA in the blastula.

8.2.1. Speculations on the mechanism by which nuclear localisation of XrelA is controlled in the blastula.

Any speculations as to the mechanism of nuclear localisation of XrelA in the blastula are necessarily based on purely circumstantial evidence. Hopefully, the value of these speculations is that they can provide testable hypotheses. In this section two lines of evidence are described which suggest possible links between FGF signalling in the blastula and nuclear localisation of XrelA.

Models of the mechanism by which the FGF signal is transduced leading to the formation of mesoderm, provide a link with the models of activation of NF-κB. FGF receptors belong to the tyrosine kinase family of transmembrane receptors. The paradigm for the transduction of signals from receptors of this family involves ligand dependent dimerisation and autophosphorylation on multiple tyrosine residues. When phosphorylated, these tyrosine residues become binding sites for SH2 domain containing proteins (reviewed in Cohen et al., 1995). Co-immunoprecipitation experiments using *Xenopus* blastula extracts and an antibody against *Xenopus* FGFR-1 identified complexes of this receptor with various SH2 domain containing proteins in the blastula including Grb2, as well as a protein known to associate with Grb2 in other systems called Sos1 (Ryan and Gillespie, 1994). Sos1 is a guanine nucleotide exchange factor which is known to be an upstream activator of Ras in various systems (reviewed in McCormick, 1993). Increasing evidence points to the importance of this pathway in the transduction of mesoderm inducing signals from the FGF receptor. Dominant inhibitory forms of ras and raf[21] have been shown to inhibit mesoderm induction by FGF in caps, and to produce phenotypes very similar to the XFD phenotype when expressed in whole embryos (Whitman and Melton, 1992; MacNicoll *et al.*, 1993). Overexpression of wild-type ras and raf, however, can induce mesoderm formation in animal caps. One of the downstream effects of raf activation is the activation of MAP kinase (MAPK) by phosphorylation, via a kinase cascade (reviewed in Nishida and Gotoh, 1993). Activation of MAPK by overexpression of a kinase involved in this cascade, called MEK, can also induce mesoderm formation in animal caps (LaBonne *et al.*, 1995). The model of FGF

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21 As discussed in chapter 5 (section 5.1) the targets for these dominant negatives are not ras and raf themselves, but their upstream activators (in the case of raf, one of these at least is ras), so an element of caution is required in interpreting results obtained with these clones.

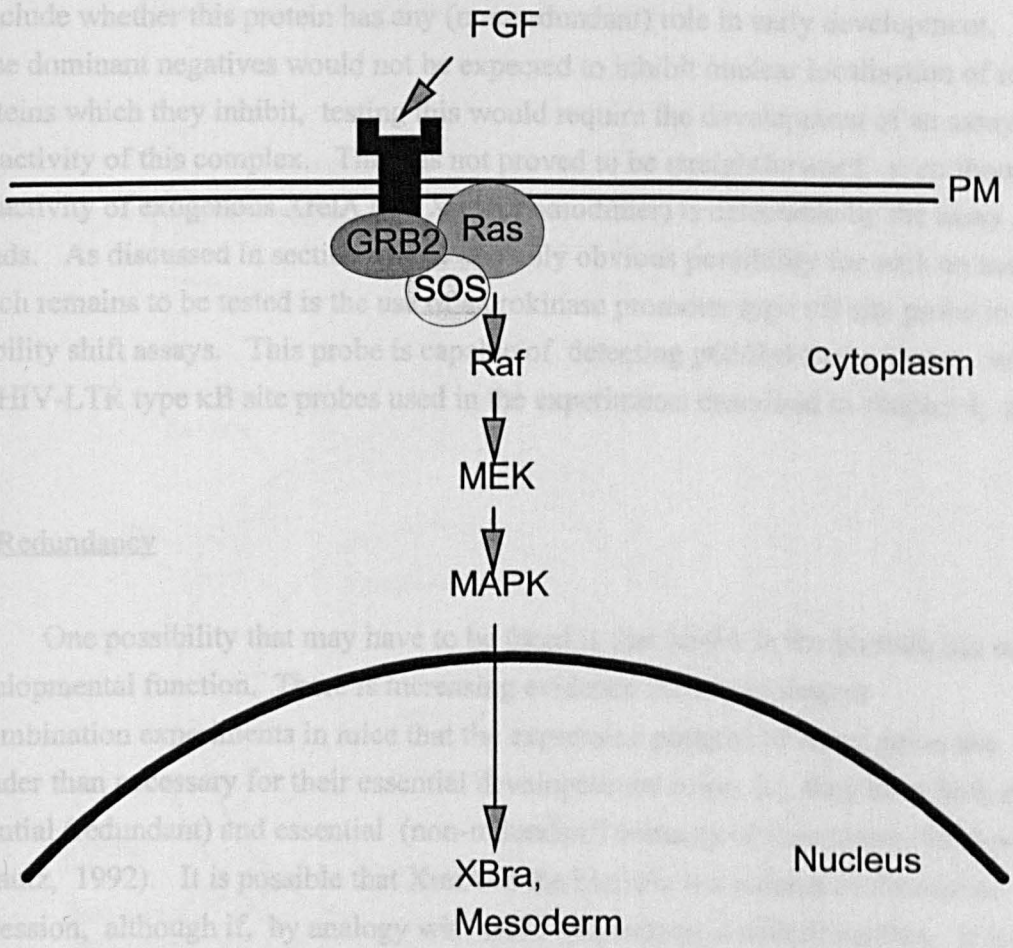
signal transduction leading to mesoderm induction resulting collectively from this data is shown in figure 46.

As discussed in the introduction (see figure 2), NF-κB activation by a number of stimuli is now thought to involve raf acting serially, or in parallel with a pathway involving ceramide as a second messenger. Overexpression of an activated form of raf has been shown to be sufficient for activation of NF-κB, at least in some cell lines. However, the fact that activation of raf does not always lead to activation of NF-κB suggests that this effect is limited by context, e.g. cell type or the presence of additional, synergising signals. Raf could potentially provide a means by which the nuclear localisation of an XrelA containing complex could be controlled by FGF.

The other piece of circumstantial evidence for FGF control of XrelA nuclear localisation is that the predicted distribution of active FGF in the blastula corresponds to the pattern of nuclear localisation of XrelA. The evidence for this predicted pattern is discussed in detail in the introduction (see page 44; also see figure 12), but I will summarise it again here. Animal caps contain sub-inducing levels of active MAPK, which can be inhibited by XFD expression (LaBonne et al., 1995) indicating that blastula animal caps contain sub-inducing levels of FGF. The fact that induction of some immediate early genes by activin in animal caps is inhibited by XFD suggests that these sub-inducing levels of FGF are required for animal caps to be competent to respond to activin. Recently FGF has been shown to be capable of inducing mesoderm formation in vegetal explants at levels of treatment which would be sub-inducing for animal caps (Cornell et al., 1995). This has been interpreted as implying that FGF treatment in these experiments is acting as a competence factor for the 'activin-like' mesoderm inducing factors secreted by vegetal cells. If this is the case then exclusion of FGF from the vegetal hemisphere could be an explanation of why the vegetal hemisphere does not induce itself to form mesoderm.

The hypothesis that FGF signalling is necessary for activation (and therefore nuclear localisation) of an XrelA containing complex in the blastula could be easily tested by using the antiserum raised by Elaine Bearer to investigate the effects of expressing XFD on the nuclear localisation of XrelA protein.

FIG. 47:- Transduction of mesoderm inducing signals from the FGF receptor.



8.2.2. Potential roles for XrelA in the blastula

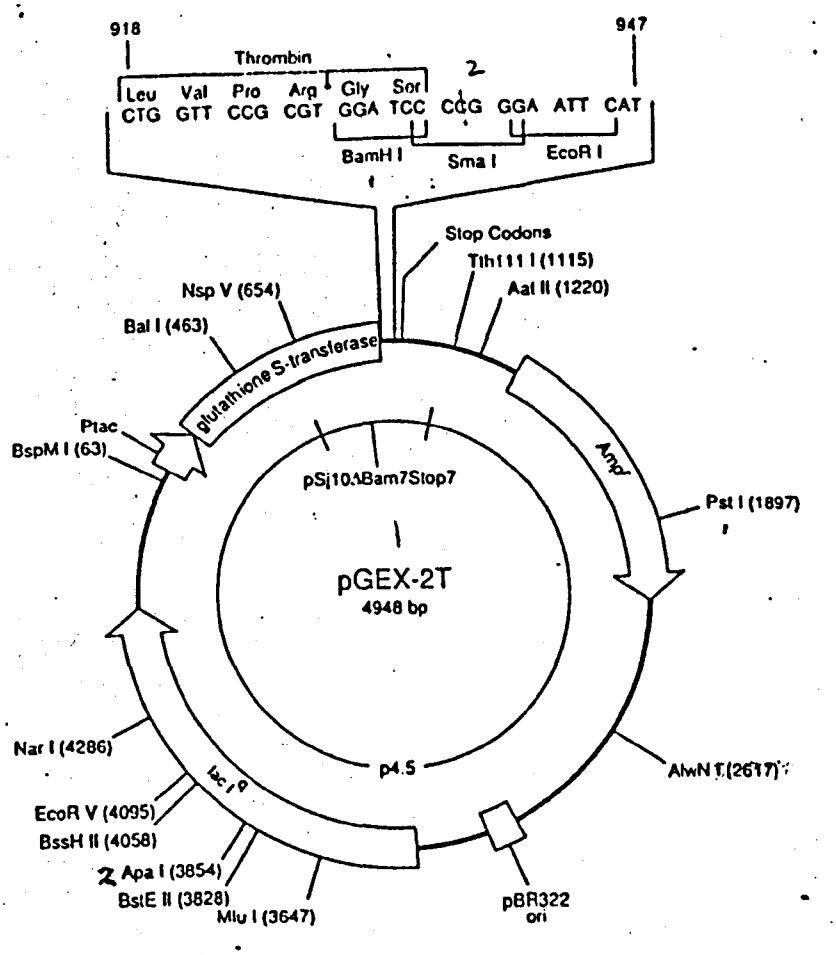
Until it is known whether the dominant negatives p50ΔSP and XrelAΔSP222 are inhibiting the activity of nuclear localised XrelA in the blastula it is not possible to conclude whether this protein has any (non-redundant) role in early development. As these dominant negatives would not be expected to inhibit nuclear localisation of rel proteins which they inhibit, testing this would require the development of an assay for the activity of this complex. This has not proved to be straightforward, even though the activity of exogenous XrelA (i.e. XrelA homodimer) is detectable by the assay as it stands. As discussed in section 4.3.1., the only obvious possibility for such an assay which remains to be tested is the use of a urokinase promoter type κB site probe in gel mobility shift assays. This probe is capable of detecting p65/Rel homodimers, which the HIV-LTR type κB site probes used in the experiments described in chapter 4, are not.

Redundancy

One possibility that may have to be faced is that XrelA in the blastula has no developmental function. There is increasing evidence from homologous recombination experiments in mice that the expression patterns of many genes are broader than necessary for their essential developmental roles, i.e. they have both non essential (redundant) and essential (non-redundant) domains of expression (Reviewed in Tautz, 1992). It is possible that XrelA in the blastula is a redundant domain of expression, although if, by analogy with relA, expression is ubiquitous then, it is the localisation event which would be redundant if XrelA has no essential development role.

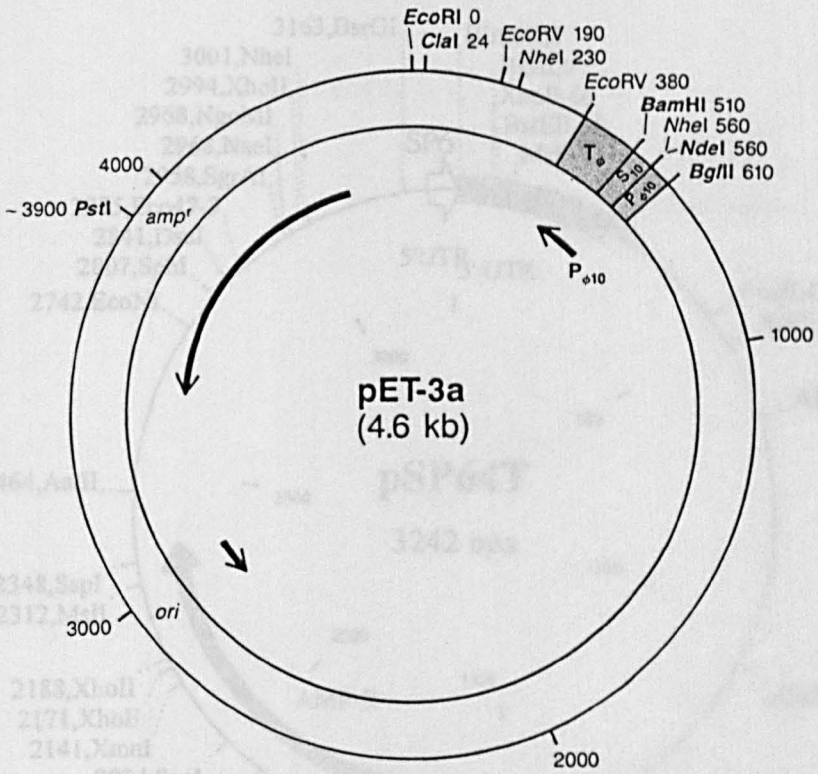
Appendix 1:- Plasmid Vectors.

pGEX-2T Expression Vector

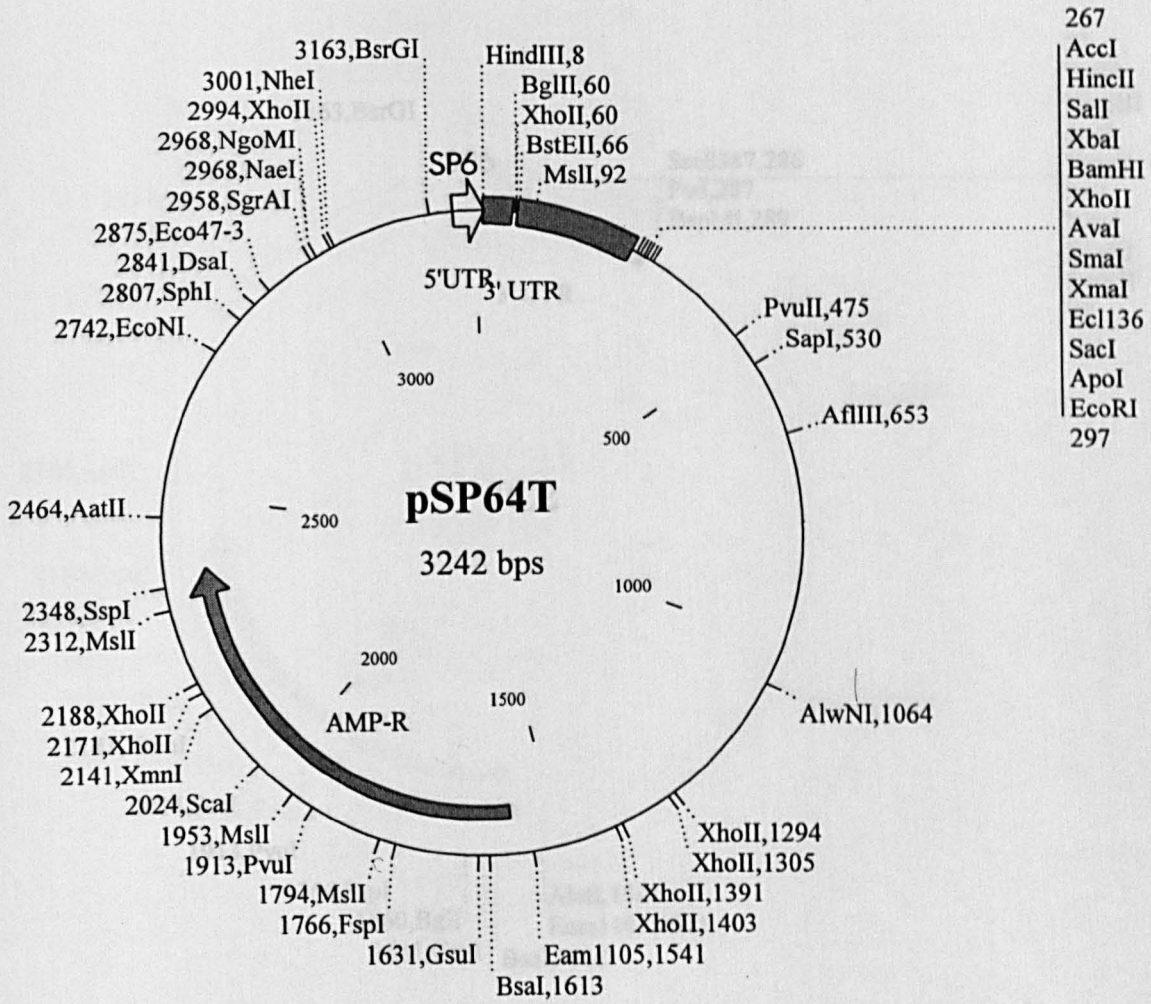


Structure of the expression vector pGEX-2T (Pharmacia) in which proteins can be fused to a glutathione S transferase affinity tail. The expressed protein fusion is purified using glutathione sepharose and the presence of the thrombin cleavage site enables subsequent removal of the protein of interest from the GST tail.

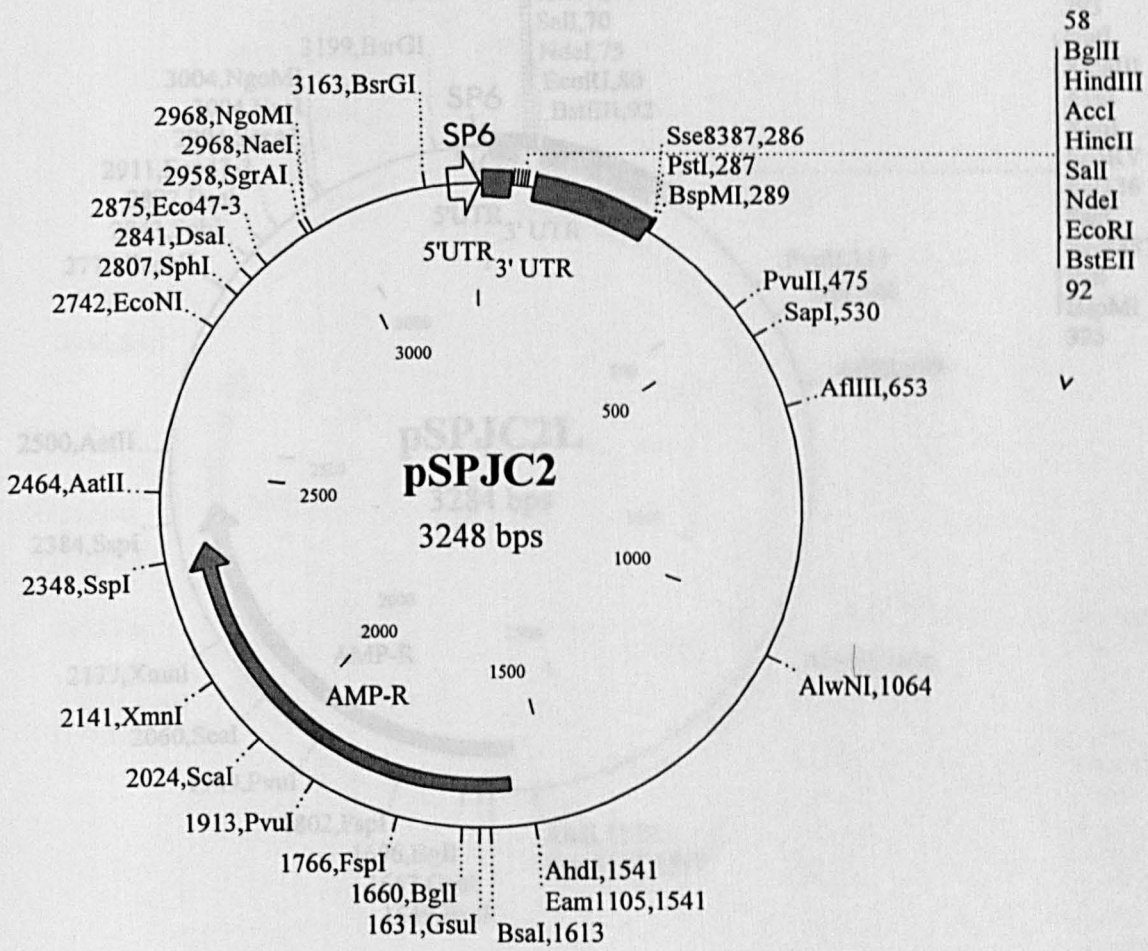
pET-3a Expression Vector



pET-3 carries the bacteriophage T7 ϕ10 promotor and terminator. The terminator may make the transcripts more resistant to exonucleolytic degradation (Studier and Moffatt, 1986). pET-3a is a derivative of pET-3 into which the translation start of bacteriophage T7 ϕ10 with a BamH1 site at codon 11 has been inserted. The NdeI site (GATATG) is located at the translation start site and can be used to construct a plasmid that directs the expression of native proteins. For more information see Sambrook *et al.*, 1989.



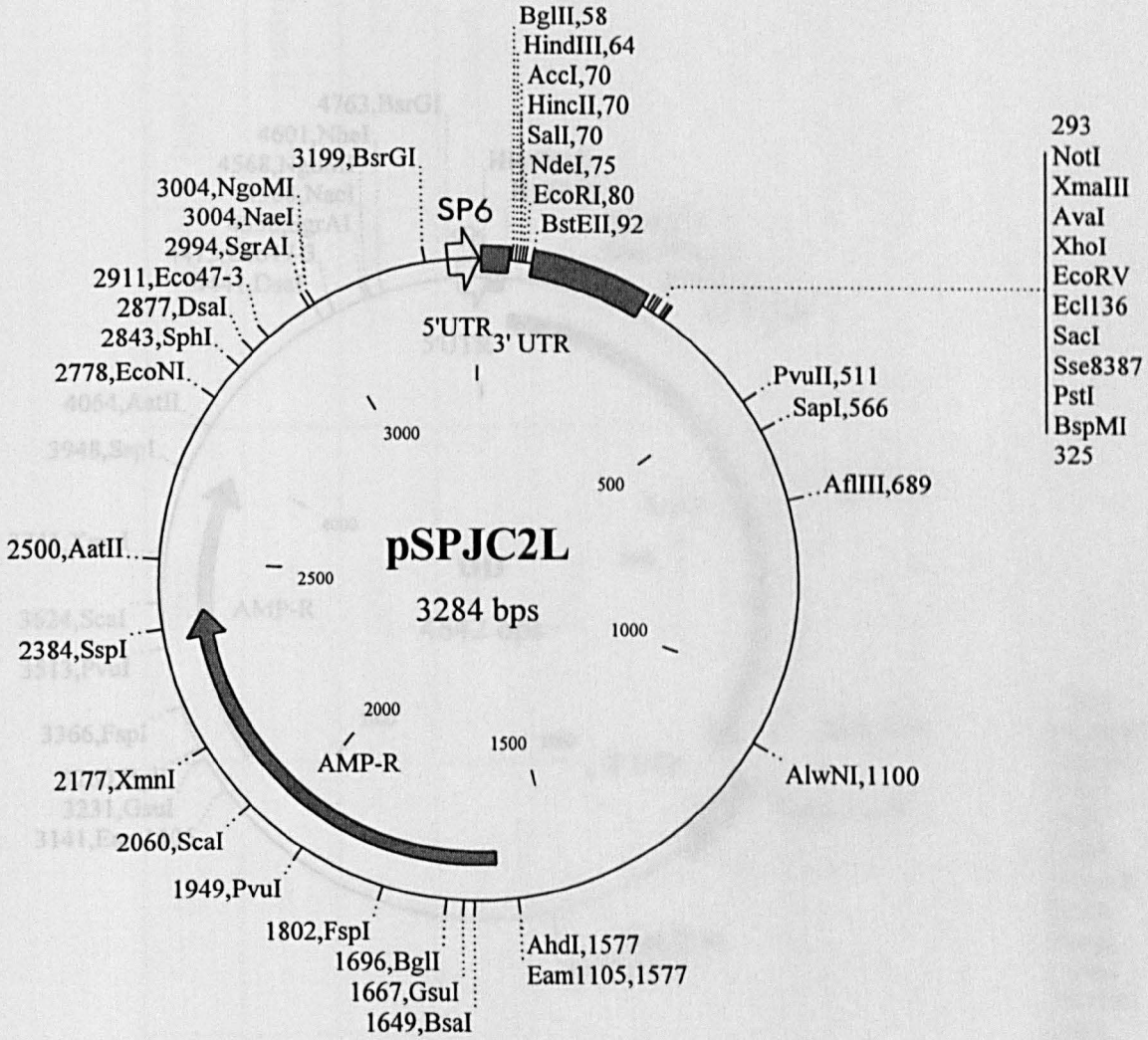
The transcription vector pSP64T allows transcription of stable mRNA, using SP6 RNA polymerase, for injection into Xenopus oocytes or embryos. It was originally derived from transcription vector pSP64, as described in Krieg and Melton (1984) and contains the 5' and 3' UTR of the *Xenopus β-globin* transcript, separated by a Bgl II site to allow subcloning of the desired ORF.



pSPJC2 is a derivative of pSP64T (Cooke, unpublished) which lacks the old pSP64 multi-cloning site as well as the Hind III site adjacent to the SP6 promoter of pSP64T, and has a new multi-cloning site between the *β-globin* UTR's

Linker sequence:-

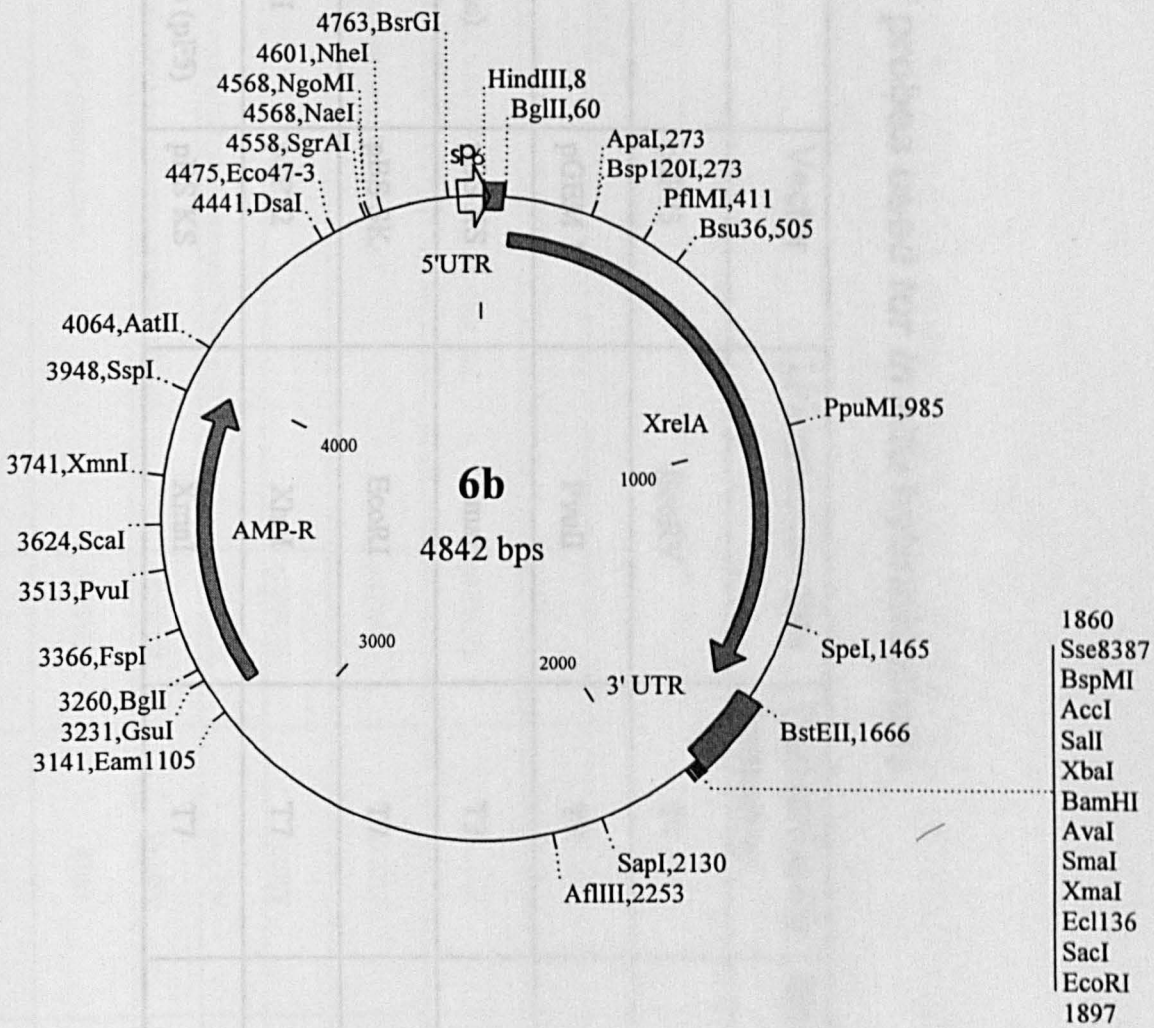
```
          EcoRI     NdeI      HincII  HindIII
5'  GATCCGAATTCATATGTCGACCAAGCTTA   3'
      CGTTAAGTATACAGCTGGTTCGAATCTAG
```



pSPJC2L is a derivative of pSPJC2 containing a new multi-linearisation site adjacent to the *β-globin* 3' UTR.

Linker sequence:-

```
                 XhoI/
        NotI     AvaI        EcoRV              SacI
5'      AGCGGCCGCTCGAGATATCGGACCTGAGCTCCTGCA   3'
        ACGTTCGCCGGCGAGCTCTATAGCCTGGACTCGAGG
```



6b consists of pSP64T containing the complete ORF of XrelA subcloned into the Bgl II site (Richardson *et al.*, 1994)

Appendix 2 :- Transcription constructs.

Table of probes used for *in situ* hybridisations.

Name	Vector	Linearisation Site	Transcribe for Antisense	Size (Kb)	Reference
Xbra	pSP73	EcoRV	T7	2.4	Smith et al1991
Xwnt-8	pGEM 1	PvuII	T7	0.9	Smith and Harland, 1991
Gsc (pDGsc)	pBS KS II	SmaI	T3	0.5	Cho et al1991
Xnot.2	pBS SK	EcoRI	T7	1.5	Von Dassow et al1992
Collagen II	pSP72	XhoI	T7	0.5	Amaya et al1993
Pintallavis (pF5)	pBS KS	XmnI	T7	1.8	Ruiz i Altaba and Jessel, 1992

Table of contructs used to make synthetic mRNA for overexpression experiments.

Name	Description	Plasmid	Cut Site for SP6 Transcription	Observed Protein Size	Reference
XrelA	Xenopus relA (p65) homologue	pSP64T	EcoRI	68	Richardson *et al.*, 1994
XrelAΔ222	XrelA with transactivation domain deleted	pSPJC2	Pst1	38	Richardson *et al.*, 1994
XrelAΔSP	XrelA with deleted DNA binding domain	pSP64T	EcoR1	46	Richardson *et al.*, 1994
XrelAΔ222SP	XrelA with deleted DNA binding and transactivation domains	pSPJC2L	EcoR1	18	See Chapter 5
Xrel2.	Xenopus c-rel homologue	pSP64T	EcoR1	68	David Tannahill, unpublished.
p105	Human NFKB1 (p105)	pSPJC2L	EcoRV	110	See Chapter 5
p50	p50 equivalent, reconstructed from p105	pSPJC2L	EcoRV	63	See Chapter 5
p50ΔSP	p50 with transactivation domain deleted	pSPJC2L	EcoRV	38	See Chapter.5
Nuclear β-gal	nuclear β-galactosidase	pGEM3z	XhoI		Smith and Harland, 1991
XFD	Truncated Xenopus FGF receptor dominant negative.	pSP64T*	EcoR1	46	Amaya *et al.*, 1991

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